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ALPHA-METHYLDOPA MELANIN

SYNTHESIS AND STABILIZATION *IN VITRO**

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Abstract—Alpha-methyldopa melanin was produced by oxidation of α-methyldopa in an alkaline medium. The progression of the oxidation and its susceptibility to interruption by chelation with borate ion were investigated. The oxidative product resulting from this chelation with borate ion was shown to be of macromolecular dimensions, unable to further incorporate ^{14}C-labeled alpha-methyldopa, resistant to further oxidation, and soluble for extended periods of time.

POSITIVE anti-globulin tests and hemolytic anemia in patients receiving penicillin have been associated with antibodies whose specificity is directed toward the drug or its metabolic products, both of which may be haptens.[1,2] Recently, positive anti-globulin tests, and in some instances hemolytic anemia, have been reported in patients treated with the antihypertensive agent, alpha-methyldihydroxyphenylalanine (α-methyldopa).

The nature of the serologic changes has been investigated. They may be summarized as follows: (1) serologic changes appear unrelated to the chemical mechanism of the antihypertensive effects of the drug[3] and do not depend on interaction between the antibody and alpha-methyldopa, its congeners or metabolites;[4,5] (2) alpha methyldopa is not haptenic, and antibody binding to red cells is not dependent on the presence of the compound;[6] (3) the antibodies eluted from the anti-globulin positive red cells are often specific for a well defined Rh antigen.

Some data suggest that incubation of the drug in whole blood with oxidation of the alpha-methyldopa produces a positive anti-globulin test. Prolonged incubation of whole blood with the compound can produce red cell agglutination.[6] The hypothesis that oxidation products may be antigenic themselves (as in the case for similar polymers of epinephrine[7]) or involved in the alteration of immunologic reactivity is suggested.

This report described the results of experiments designed to control the oxidation and polymerization of α-methyldopa. The objective was to produce a stable, soluble, immunologically reactive material which could be used to investigate the antigenicity of oxidation products of α-methyldopa and their possible role in production of positive anti-globulin tests in patients.

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MATERIALS AND METHODS

Rationale

The initial step in the oxidation and polymerization of the substituted catechols in the Raper–Mason[8,9] pathway (Fig. 1) is the formation of a quinone with its free radical intermediate.[10,11] Protecting the hydroxyl groups of the catechols should slow the rate of polymerization. In the present study, chelation by borate ion was selected as a means of blocking oxidation because: (1) borate reacts with ortho-dihydroxy compound in the *cis* configuration;[12,13] (2) borate chelation is a reversible reaction permitting recovery of components in their native form;[14–16] and (3) borate ion prevents oxidation of a number of mono- and disaccharides,[17,18] as well as of epinephrine.[19]

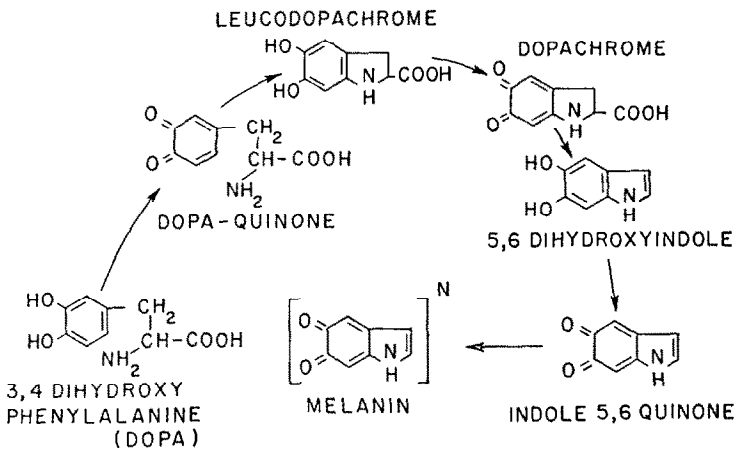

FIG. 1. (A) Raper–Mason pathway of oxidation of DOPA. It is highly probable that two or more additional intermediates exist.

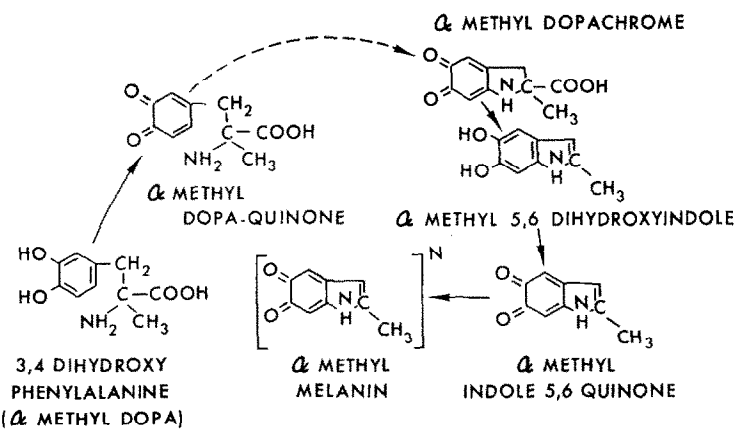

Fig. 1. (B) Possible pathway of oxidation of α-methyldopa. It is probable that the α-methyldopa melanin polymer differs from the melanin polymer in aspects other than those shown.

Materials

Stock solutions (0·025 M) of α-methyldopa* and L-dihydroxyphenylalanine† (DOPA) in 0·15 M unbuffered sodium chloride were prepared and stored at 4° under nitrogen in opaque vials. All dilutions of stock solutions were made with 0·15 M sodium chloride buffered with 0·02 M Tris and adjusted to the appropriate pH with hydrochloric acid. Solutions of 0·5 M sodium borate, ranging in pH from 5 to 10, were prepared by mixing appropriate amounts of 10 M sodium hydroxide, boric acid and water.

Methods

Ultraviolet and visible absorption spectra were determined with a Cary recording spectrophotometer, model 14. In kinetic studies, a Hitachi–Perkin Elmer spectrophotometer (model 139) was used to determine changes in absorbancy. For radioactive studies, a mixture of α-methyldopa and ^{14}C-labeled α-methyldopa‡ (specific activity, 5×10^{-3} μc/m-mole) was used. Assays were performed with a Packard Tri-Carb liquid scintillation counter, model 527.

The products were dialyzed in washed cellophane tubing (0·6 cm in diameter) against two changes of a 10-fold volume excess of 0·5 M sodium borate, pH 6, at 4° for 16 hr.

Gel filtration was performed at room temperature using a Sephadex G-100 column (100 × 2·5 cm) calibrated with bovine serum albumin, and using 0·15 M sodium chloride, buffered with 0·02 M, in Tris-HCl, pH 8·0, as eluant. Exclusion volume was determined using Dextran 2000 (Pharmacia, Uppsala).

The dependence of the rate of oxidation on pH was studied by measuring the optical density at 475 mμ of solutions of DOPA and α-methyldopa at pH levels ranging from 5 to 10 after incubation for 1 hr in light and air.

The effect of borate ion on the rate or extent of oxidation was assessed, and polymerization of α-methyldopa was studied in the presence of various concentrations of sodium borate at pH values ranging from 6 to 10. The optical density at 475 mμ was taken as a measure of the extent of the reaction. The effect of borate ion on the oxidation and polymerization of α-methyldopa was further assessed as follows. Control and reaction solutions of 0·2 mM α-methyldopa in Tris-saline buffer, pH 1, were prepared, and oxidation was allowed to proceed for 15 min. After 15 min, 0·02 M Tris-saline, pH 10, was added to the control solution and 0·5 M sodium borate, pH 6, to the reaction mixture.

Kinetic studies were carried out with solutions of 0·1 mM α-methyldopa and DOPA in Tris-saline, pH 10; changes in absorbance were recorded at three wavelengths representing absorption maxima for selected intermediates found at various stages in the course of oxidation.

To ascertain whether the addition of borate to oxidized α-methyldopa (see Results) produced a maximal field of oxidation products without formation of insoluble macropolymers, a mixture of α-methyldopa and ^{14}C-labeled α-methyldopa (5 mg/ml in 0·2 M Tris-saline, pH 10) was incubated at 37° for 1 hr, and an equal volume of 0·5 M sodium borate, final pH 7·2, was added. The resultant solution was dialyzed

* Aldomet, provided by Merck, Sharpe & Dohme, West Point, Pa.
† L-DOPA, Nutritional Biochemical Corp., Cleveland, Ohio.
‡ Provided by Dr. Meriweather, Merck, Sharpe & Dohme, West Point, Pa.

against 10- to 20-fold volumes of 0·5 M borate, pH 6, in the dark for 18 hr at 4°, then dialyzed against phosphate-buffered saline, pH 7·4, and the per cent radioactivity recovered in the dialysate measured. The approximate size of the material in the dialysate was then determined by gel filtration on a Sephadex G-100 column calibrated with bovine serum albumin.

RESULTS

In preliminary studies, the spectrophotometric characteristics of α-methyldopa were compared with those of DOPA.[16] The additional α-methyl group did not significantly alter the reactivity of the catechol ring. A roughly linear relationship was found between rate of oxidation and pH for both DOPA and its α-methyl derivative at pH 5–9 (Fig. 2). Solutions developed a red tint within the first few minutes and became progressively darker as the reaction proceeded. No increase in optical density occurred between pH 9 and 10, suggesting that at pH 9 or greater all initial reactant is converted to the intermediates within 1 hr. Incubation for longer periods resulted in further darkening of solutions and the eventual formation of an insoluble black precipitate.

The ultraviolet and visible spectra of α-methyldopa and DOPA solutions were recorded at 15-min intervals during the course of oxidation. By 2 min, the maximum absorption occurred at 290 mμ. The spectral changes of α-methyldopa (Fig. 3) were indistinguishable from those of DOPA.

As shown in Fig. 4, 0·5 M borate almost completely inhibited oxidation and polymerization of α-methyldopa at all pH values. At concentrations of 0·25 and 0·12 M borate, there was slight reactivity, which was inversely proportional to the concentration, but the degree of inhibition was equal at all pH levels. At concentrations of less than 0·25 M, however, the degree of inhibition was inversely proportional to the pH. At pH 6 and 7, moderate concentrations appeared to be less inhibitory than low concentrations, but this difference may not be significant.

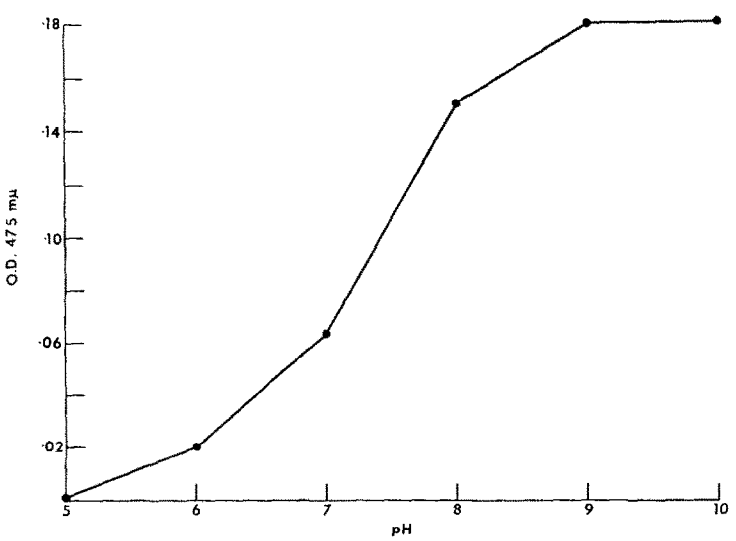

FIG. 2. Plot of optical density at 475 mμ versus pH after incubation of 0·0125 M α-methyldopa in 0·15 M sodium chloride–0·25 M sodium borate in light and air for 1 hr.

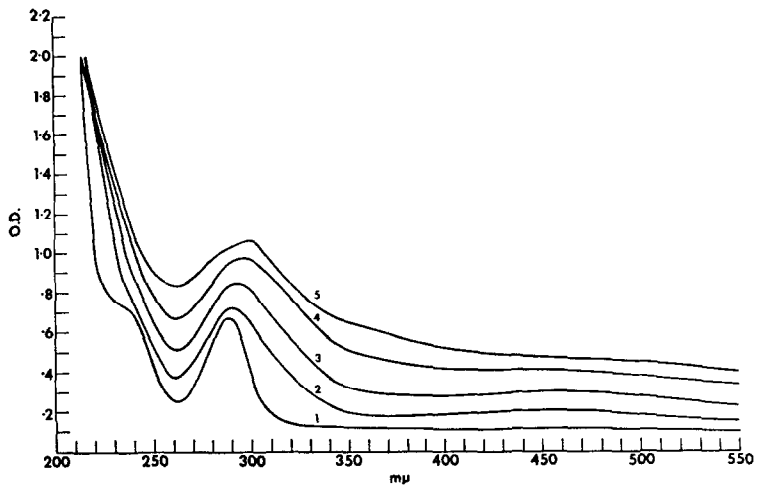

FIG. 3. Ultraviolet and visible spectra of α-methyldopa during the course of oxidation at pH 10. 1 = at 2 min, 2 = at 15 min, 3 = at 30 min, 4 = at 45 min, and 5 = at 60 min. Spectral shift had occurred within 2 min, so that the absorption maximum is at 290 mμ.

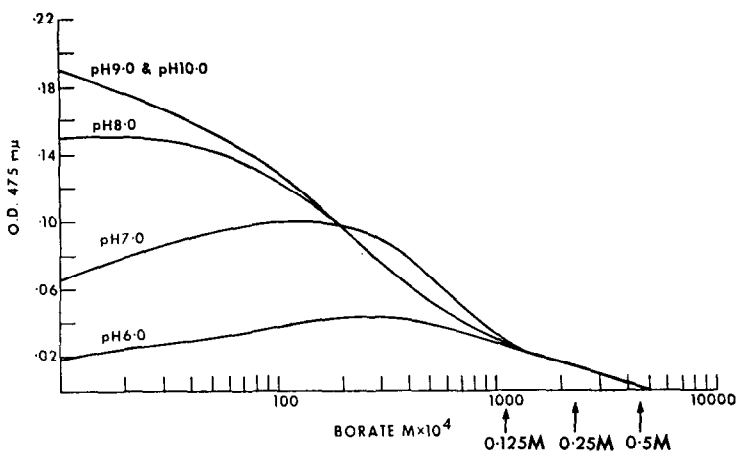

FIG. 4. Plot of optical density of 0·125 M α-methyldopa at 475 mμ versus concentration of sodium borate at pH 6–10.

When kinetic studies were carried out with α-methyldopa and DOPA and changes in absorbance were recorded at the three wavelengths representing absorption maxima for selected intermediates in the course of oxidation, three different reaction rates were observed (Fig. 5). The curve at 290 mμ represented the absorbance of α-methyldopa quinone, the first intermediate in monomeric oxidation, plus 5,6-dihydroxyindole, the last intermediate. The curve at 275 mμ represented the absorbance by α-methyldopa and the 5,6-dihydroxyindole form of α-methyldopa. The curve at 475 mμ represented the absorbance of α-methyldopa chrome, a middle-stage intermediate product. The maximum absorbance at 290 mμ occurred at 45 min, suggesting that formation of α-methyldopa quinone is rapid compared with formation of α-methyldopa chrome.

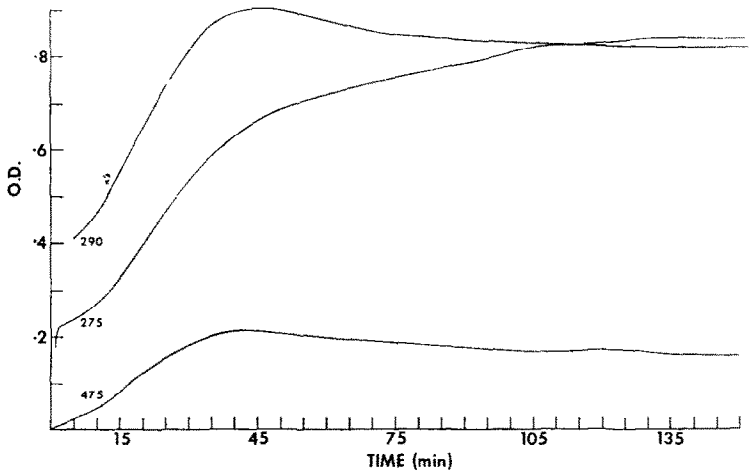

FIG. 5. Plot of optical density at 275, 290 and 475 mμ as a function of time during the course of oxidation of α-methyldopa at pH 10.

Since the decrease in absorbance at 290 mμ was paralleled by a decrease at 475 mμ, the rate-limiting step is the conversion of α-methyldopa chrome to 5,6-dihydroxyindole.

The curve of absorbance at 275 mμ had three phases (Fig. 5). The first phase, with a large positive slope, represented the absorbance contributed by the formation of α-methyldopa quinone; after a few minutes this was augmented by the absorbance due to formation of 5,6-dihydroxyindole. The second phase, which began when the α-methyldopa quinone curve (290 mμ) reached its maximum, had a smaller positive slope and represented only the absorbance by 5,6-dihydroxyindole. The third phase, which had a slope of zero, represented the equilibrium between the formation of 5,6-dihydroxyindole and its incorporation into the polymer.

As shown in Fig. 6, addition of Tris-saline (control solution) to a reaction mixture containing 0·2 mM α-methyldopa resulted in formation of α-methyldopa chrome, a middle-stage intermediate product (curve at 475 mμ); in contrast, the addition of 0·5 M sodium borate to the reaction mixture halted α-methyldopa oxidation and caused conversion of preformed α-methyldopa to 5,6-dihydroxyindole (curve at 275 mμ). Changes in the pH of the sodium borate solution between 6 and 10 did not alter the effect of the borate ion. This observation together with the results obtained by varying the concentration of sodium borate (Fig. 4), suggested that oxidation of α-methyldopa at pH 10 for 1 hr and termination of the reaction by the addition of borate would result in a maximum yield of oxidation products without formation of insoluble macropolymers.

This hypothesis was tested by incubating a mixture of α-methyldopa and ^{14}C-labeled α-methyldopa (in 0·2 M Tris-saline) at 37° for 1 hr. The resultant solution was brownish-black and contained no precipitate. When an equal volume of 0·5 M sodium borate was added, final pH 7·2, the solution turned purple-black. After sequential dialysis against borate and buffered saline (see Methods), the nondialyzable fraction was considerably darker; however, the first dialysate was black as well, therefore suggesting that at least two species of polymer were produced by such treatment, one dialyzable and the other not so. About 40 per cent of the radioactivity was consistently

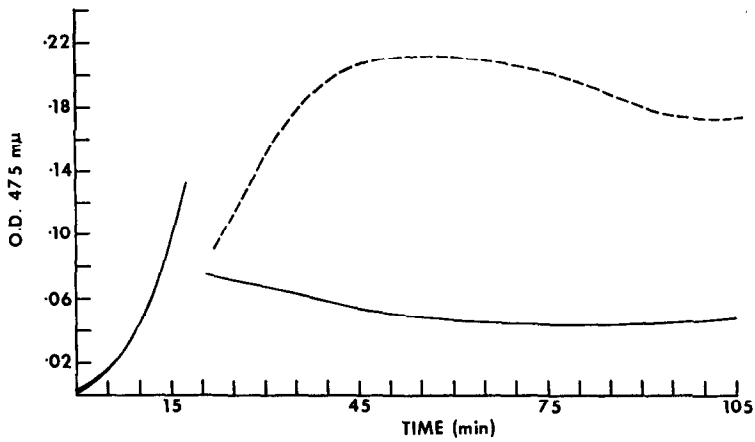

FIG. 6. Change in optical density at 475 mμ effected by the addition of 0·5 M sodium borate, pH 6, to 0·02 mM α-methyldopa in Tris-saline during the course of oxidation. Dotted line = control solution; solid line = reaction mixture. The break in the curves at 15 min represents the effect of dilution by the addition of sodium borate to the reaction mixture or of 0·02 M Tris-saline, pH 10, to the control mixture.

recovered in the dialysate. Exposure of the reactants to borate for varying periods before dialysis did not affect the recovery of labeled material in the dialysate. This finding suggests that polymerization either occurs as soon as the intermediate products are formed[20,21] or proceeds through a mechanism that is inhibited by borate ion.

Gel filtration of the nondialyzable fraction Sephadex G-100 gave a broad elution pattern starting in the area of the albumin peak (50 per cent V_t) and extending throughout the inner volume of the column (Fig. 7). The elution curve showed no distinct peaks, but most of the material had an elution volume greater than that of serum albumin with its peak centered at 50 per cent V_t. The polymer of α-methyldopa showed relative maxima at 64 and 84 per cent V_t, with a shoulder at 90 per cent V_t. A portion of the sample apparently became bound to the Sephadex beads in the first few inches of column length and a small amount of this material continued to be eluted very slowly even beyond 150 per cent V_t.

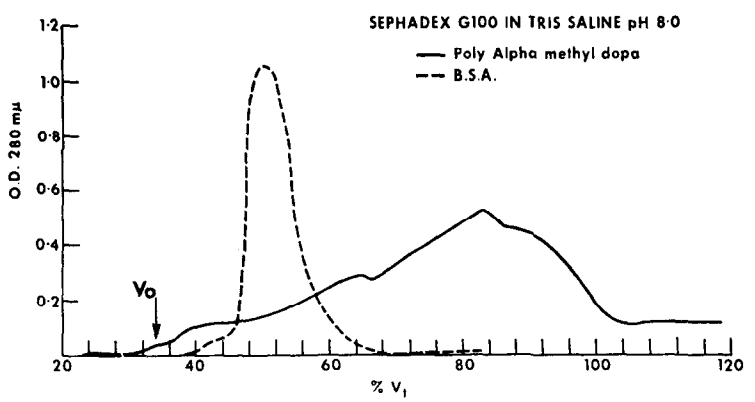

FIG. 7. Chromatogram of α-methyldopa polymer on a Sephadex G-100 column calibrated with bovine serum albumin (BSA). Arrow indicates V_0.

The nondialyzable fraction was stable for 2–3 weeks at room temperature in the presence of light and air. Storage for longer periods resulted in the formation of a black precipitate. As the precipitate increased in amount, the supernatant became progressively lighter. Lyophilization of the solution and storage of the material for varying periods did not diminish solubility.

DISCUSSION

Comparison of DOPA and α-methyldopa in the reactions investigated in this study ndicates that the presence of the methyl group on the alpha carbon does not affect the ability of the compound to enter the melanin pathway. This pathway requires an enzymatic step for the oxidation of DOPA to DOPA-quinone *in vivo*,[8] but not in systems *in vitro*.[9]

From the shape of the curve obtained in the pH study (Fig. 2), the estimated pK of α-methyldopa is about 8·0, which is close to the pK of 8·7 for the metahydroxyl group of phenylephrine.[22] The flattening of the curve at pH 9 suggests either that all starting product had passed through the early intermediate phase within the incubation period or that the rate of formation of the dopa chrome intermediate and 5,6-dihydroxyindole is the same. The former explanation is favored by the data obtained in the kinetic studies (Fig. 6), which indicate that conversion of the product absorbing at 475 mμ (dopa chrome) to the next intermediate (5,6-dihydroxyindole) occurs at a slower rate than conversion of the preceding intermediate (dopa quinone). In addition, the disappearance of the relative absorption maximum at 475 mμ indicates that the absorbed material was being consumed. These data suggest that at pH 10 the conversion of starting product to dopa chrome or an intermediate in the fast phase of polymerization reaction [8,23] is completed within 1 hr.

The lesser inhibition of the reaction at low concentrations of borate ion at pH 6 and 7 (Fig. 4) suggests possible differences in the binding constants of the various borate–diol complexes (Fig. 8).[24,25] Assuming that the pK of α-methyldopa is about 8, then the low pH samples are relatively undissociated, and in low concentrations of borate ion, a 1:1 ratio of chelation is favored. With moderate concentration, the ratio is probably two borate to one diol. Since the binding constant (K) of B + M →

The interaction between polyols and boric acid can be formulated as follows:

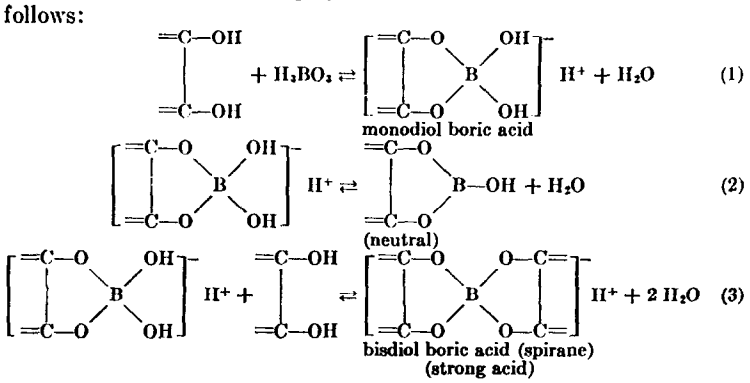

FIG. 8. Three possible forms of polyolborate complex. Reproduced from Böeseken[12] by permission of the author and publisher.

BM is only 1 per cent that of the K of B + 2M → BM_2,[14] some dissociation of BM → M + B is likely (pH 6 and pH 8, Fig. 4). The unchelated diol is then in the ionic form and available for oxidation. In samples at high pH, the inhibition of the reaction was approximately in a linear relationship to the concentration of borate ion.

Comparison of the ultraviolet and visible absorption spectra of borate-treated and untreated α-methyldopa oxidized for various periods at pH values ranging from 6 to 10 shows that the effect of borate was not due merely to production of a leukodopachrome. The spectra did not differ; however, the absorption at the maximum was greater at the high pH. Such an extinction increment has been shown to result from the presence of a greater proportion of ionized forms.

The effect of borate ion on the oxidation of α-methyldopa (Fig. 7) reflects two factors: (1) borate ion prevents the further formation of the intermediate product; and (2) borate ion does not prevent an already formed intermediate from being converted to the next product. In the dialysis studies with ^{14}C-labeled α-methyldopa, the amount of labeled material in the dialysate was not markedly reduced by exposure to borate for increasing lengths of time. Thus, once borate is added, little new polymer is formed. The slow decrease in the dopa chrome form, therefore, reflects the conversion to borate-chelated 5,6-dihydroxyindole rather than incorporation of further intermediates into the polymer. According to these results, the quinone form is required for the entry of the intermediate product into the polymer. This concept is in agreement with the suggestion by Blois* and by Piatelli *et al.*[26] that any intermediate may enter the polymer, but to do so it must be in the quinone form.[23] As further evidence, the dialysate in ^{14}C studies acquired the purple-black tint which would be expected if melanochrome, the penultimate step in the melanin pathway, were free in solution. Since the next step in the reaction sequence is not the slowest one, the presence of melanochrome would indicate that borate interrupts oxidation at all stages in the reaction. Alternatively, the dark material in the dialysate could be a species of polymer of a size not retained by the dialysis tubing.

The results of gel filtration are difficult to interpret, since the polymer would not necessarily behave like globular proteins. Also, quinones may react with cross-linked dextrans and therefore might not be eluted as expected. Assuming that Andrews' plot[20,21] showing linearity of V_e versus log molecular weight can be applied to the polymer, the three peaks represent molecular weights 45,000, 12,000 and 8000. These assumed molecular weights are not consistent, however, with Berggard's[27] finding of 14,000 as a minimum molecular weight for nondialyzable material. The variance in behaviour of the polymer and that of globular proteins may result from its shape. This may be inferred from Andrews' plot,[20] since proteins with frictional coefficients outside the range of 1·1 to 1·3 fall in the area of linearity, but fall off the curve. Since the polymer of α-methyldopa is a large flat molecule, its axial ratio is far from unity, and hence its frictional coefficient is probably far from the range of 1·1 to 1·3. Thus, the elution curve obtained may reflect heterogeneity in the size of the polymer as well as heterogeneity in affinity for cross-linked dextran.

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* M. S. Blois, personal communication.

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