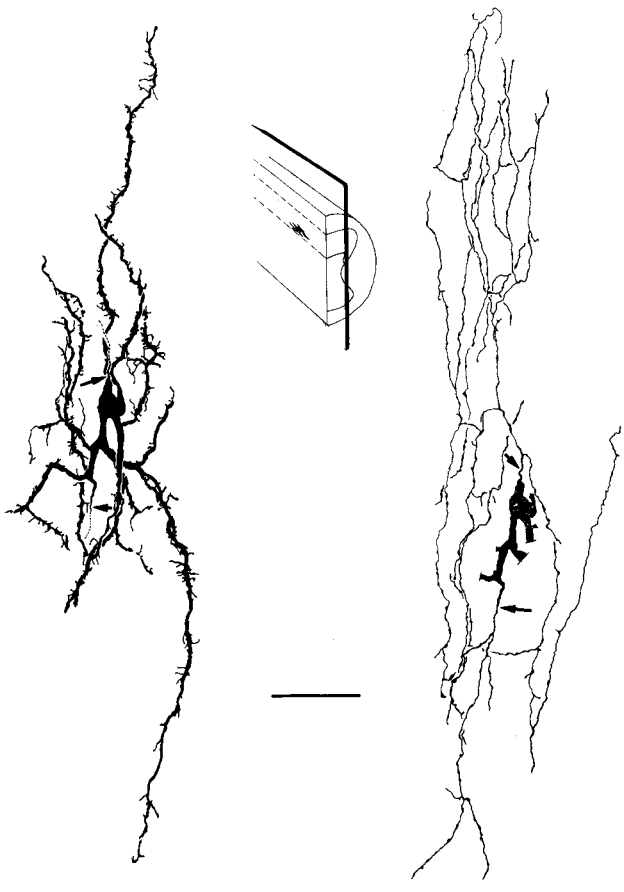

Drawing of a single SG neuron exhibiting dual axons sectioned in the sagittal plane showing the full extent of its dendritic arbor (right) and plexus generated by the 2 axons (left). Arrows indicate origin of each axon. Bar: 50 µm.

combined axis cylinder'[8] with the only difference being an axon with a separate rather than a combined origin.

In support of this contention several examples of developing neurons with 2 separate axons (destined for different white fasciculi) were observed by Ramón y Cajal[8] in the spinal cords of very early (7–9-day) chick embryos. Cajal interpreted these as an early embryonic phase of the 'cells of the combined axis-cylinder'. Based on the observation that most SG neurons in the spinal cord of the adult possess only 1 axon, it would follow that the normal course of development of these cells involves the loss of 1 axon or the fusion of the 2 axons into a single fiber. In the case of persistent dual axons in the adult, the axons could have failed to fuse or the additional axon failed to degenerate, possibly due to the establishment of sufficient connections to warrant its retention. It seems possible then, that dual axon SG cells of the adult spinal cord may represent a developmental aberrancy rather than a functionally distinct class of neurons with special physiological properties. The rarity of the dual axon cell and its structural similarity to single axon 'central' cells would seem to support this.

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Spectral properties of methemoglobins prepared by the action of sodium nitrite and potassium ferricyanide[1]

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Summary. Methemoglobin was prepared by the addition of sodium nitrite or potassium ferricyanide to oxy or deoxyhemoglobin. The spectral properties of these methemoglobins were studied before and after extensive dialysis. It is shown that the methemoglobin formed by sodium nitrite has substantial spectral differences in visible and Soret band compared to that formed by potassium ferricyanide. These differences are proportional to the excess of sodium nitrite only. This suggests that both methemoglobins are similar compounds.

Hemoglobin is oxidized by oxygen (autoxidation) as well as by a great number of oxidants such as drugs or chemicals. The most common oxidizing agents used for preparation of methemoglobin either for research or for standards in clinical laboratory are sodium nitrite and potassium ferricyanide. Although certain spectral differences between these methemoglobins have been observed, the previous studies have been made on red cells or hemolysate[3–5]. This study was undertaken to elucidate the nature of these methemoglobins in pure form in the absence of organic phosphates.

Materials and methods. Pure hemoglobin was prepared by ion exchange chromatography[6]. Hemoglobin was stripped of all organic phosphates by the method already described[7]. Spectra were recorded on a double beam Beckman spectrophotometer model 35. The oxidation was carried out at 37 °C in a 1-cm light path quartz cuvette. Hemoglobin solutions were diluted with bis-tris buffer pH.6 to a concentration of 0.2 mM. Various amounts of sodium nitrite or potassium ferricyanide were added. Adequate time was allowed to elapse until oxidation reaction was completed and the spectra were recorded. The oxidation reaction was also carried out under anaerobic condition. This was performed by placing 2 ml of hemoglobin solution in a tonometer. After complete deoxygenation by vacuum, the hemoglobin was oxidized by the injection of various

amounts of deoxygenated oxidant through a rubber stopper by a microcysringe (Corning) as has been already described[8].

Results and discussion. Figure 1 demonstrates the distinct spectral differences between acid methemoglobins prepared by sodium nitrite and potassium ferricyanide. It is to be noted that these differences are not due to pH which has been carefully checked. The spectral differences are dependent on the quantity of nitrite used for the oxidation. Figure 2 represents the difference in spectra of methemoglobin prepared in the presence of 2–20 molar excess of nitrite/heme. The spectral differences are also present at Soret band (fig. 3). When the hemoglobin is oxidized in the presence of various quantitites of ferricyanide there is no spectral difference dependent on the quantity of oxidizing agents (fig. 4). In order to see whether these changes take place during the oxidation or are merely due to the presence of nitrite, sodium nitrite in various quantities (2–1000 molar excess of nitrite/heme) was added to methemoglobin and the spectra were recorded. It is shown in figure 5 that the addition of nitrite to methemoglobin produces the same spectral changes as it does when it is added to oxyhemoglobin. The differences are due to the excess of nitrite and disappear after extensive dialysis (fig. 6). In order to demonstrate whether the changes brought about by the addition of nitrite are due to the heme binding sites or the protein moiety, potassium cya-

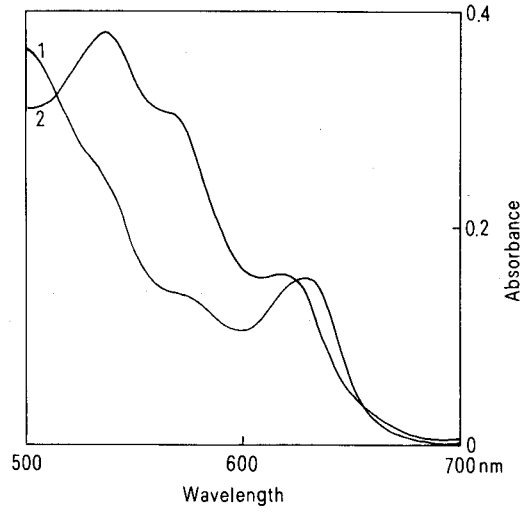

Figure 1. Spectral differences between ferricyanide methemoglobin (1) and nitrite methemoglobin (2).

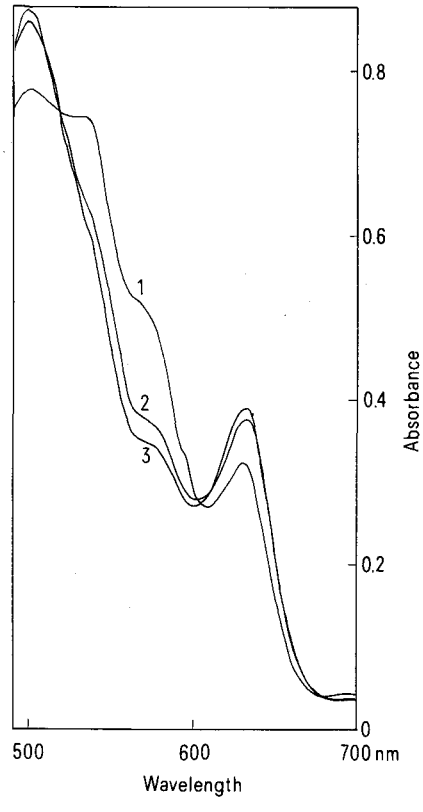

Figure 2. Oxyhemoglobin oxidation by sodium nitrite. Nitrite/heme = 2(3), 5(2) and 20 (1).

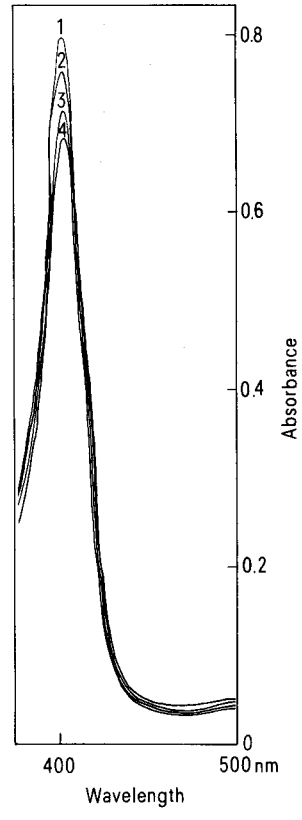

Figure 3. Spectral changes of methemoglobin in the presence of excess of sodium nitrite. No nitrite (1), nitrite/heme = 2 (2), 5 (3) and 20 (4).

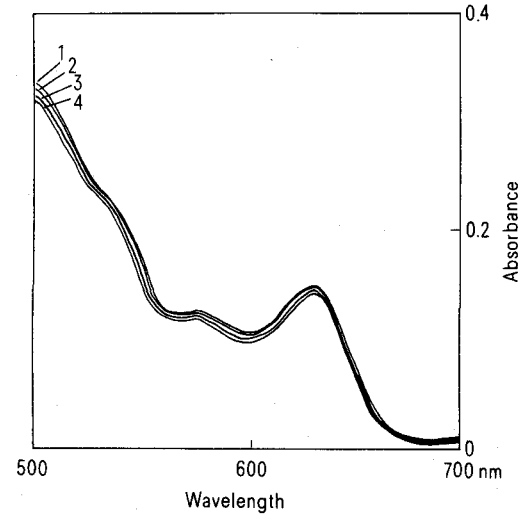

Figure 4. Spectral properties of methemoglobin prepared by the action of potassium ferricyanide. Ferricyanide/heme = 2 (1), 4 (2), 5 (3) and 10 (4).

nide was added in the presence and absence of excess of nitrite. In the presence of potassium cyanide, the excess nitrite does not cause any spectral changes indicating that the site of action is the heme and not the protein moiety.

Although the heme iron in hemoglobin is the most susceptible part of the molecule to the action of oxidants, it is conceivable that certain other sites in the protein moiety such as β-93-sulfhydryl groups, tyrosin and tryptophan residues can be oxidized modifying the properties of the molecule. However, the optical properties of heme proteins

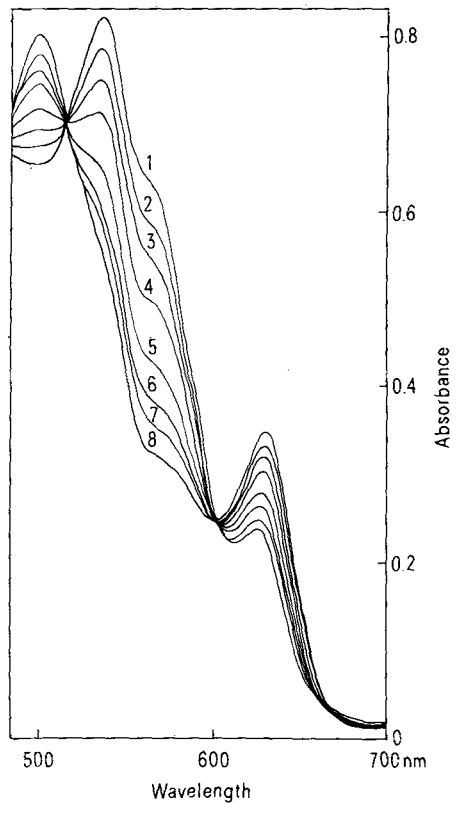

Figure 5. Spectral changes of methemoglobin in the presence of excess nitrite. Nitrite/heme = 5 (8), 10 (7), 20 (6), 50 (5), 100 (4), 250 (3), 500 (2) and 1000 (1).

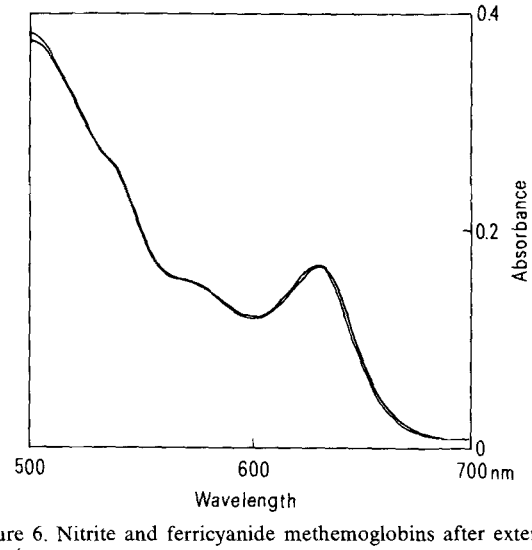

Figure 6. Nitrite and ferricyanide methemoglobins after extensive dialysis.

reflect for the most part the electronic structures of the heme rather than the protein[9]. When using different oxidizing agents, another factor which might result in methemoglobins with different properites would be specific or nonspecific binding of the oxidant to the hemoglobin molecule. In fact it has been shown that after the treatment of hemoglobin with ferricyanide, the protein appears to contain strongly bound nonheminic iron which is very difficult to remove by dialysis[10]. It has been as well shown that the properties of methemoglobin prepared by ferricyanide differ in certain details from those of the same derivative prepared by other agents[11]. On the other hand Antonini et al. on studying methemoglobin and metmyoglobin formation did not find any evidence of reaction other than heme iron oxidation[12]. In regard to heme oxidation by nitrite, there is some evidence that nitrite binds to hemoglobin prior to heme oxidation[13]. The oxidation study on pure and stripped hemoglobin by the 2 most common agents shows that the spectral changes are dependent on the excess of nitrite and not that of ferricyanide. These changes not only occur during the oxidation, but they occur also when nitrite is added to methemoglobin. The fact that after extensive dialysis the spectra of nitrite methemoglobin and ferricyanide methemoglobin are identical suggests that the spectral changes are not due to the oxidation of protein moiety and the changes are reversible. These changes cannot be due merely to the presence of nitrite but rather to binding of nitrite to hemoglobin because sodium nitrite does not have any optical absorbtion at either visible or Soret band. In view of the identical spectra of nitrite methemoglobin and ferricyanide methemoglobin in the presence of potassiums cyanide regardless of the presence of the excess oxidant, the binding of the nitrite must be at the heme site. The fact that excess of sodium nitrite does not cause any spectral changes when the heme oxidation is carried out under deoxy conditions (not shown here) suggests that the oxygen is necessary for the obserted spectral differences.

It is concluded that in the presence of oxygen, sodium nitrite forms a complex with hemoglobin which affects its spectral properties proportional to the nitrite excess. After dialysis nitrite and ferricyanide methemoglobins have identical spectra indicating indirectly that they are identical compounds.

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