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Kinetic Study of the Anaerobic Formation of Nitric Oxide Haemoglobin

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ABSTRACT

The anaerobic formation of nitric oxide haemoglobin (NOHb) has been studied in model systems containing purified sheep methaemoglobin (MetHb), nitrite and ascorbic acid.

Evidence has been obtained that the conversion of ferric pigment to the nitric oxide form proceeds through the mediation of the unstable complex nitric oxide methaemoglobin (NOMetHb) rather than via deoxyhaemoglobin (Hb).

A mechanism has been proposed which takes into account, and is in accord with, the findings of other workers in this field of study.

INTRODUCTION

Since the beginning of the twentieth century when Haldane (1901) showed that the formation of cured meat colour involved reduction of nitrate to nitrite and reaction of the latter with the muscle pigments myoglobin (Mb) and haemoglobin (Hb), considerable research has been carried out on the mechanism(s) of the reaction.

It is generally considered that the formation of the pigments responsible for the bright red colour of uncooked cured meats; namely, nitric oxide myoglobin (NOMb) and nitric oxide haemoglobin (NOHb), is associated with chemical, non-enzymatic as well as enzymatic reaction pathways (Fox & Thomson, 1963; Walters & Taylor, 1964; Walters *et al.*, 1967; Fox & Ackerman, 1968; Koizumi & Brown, 1971; Cheah, 1976).

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However, although the structures of nitric oxide myoglobin and nitric oxide haemoglobin are now firmly established (Giddings, 1977; Deatherage & Moffat, 1979), uncertainty still surrounds the mechanism(s) by which ferrous and ferric myoglobin and haemoglobin react with nitrite to produce these pigments (MacDougall *et al.*, 1975; Giddings, 1977).

Indeed, much controversy arises from the concept that the reduction of the ferric form of the pigments is a step in the sequence of reactions whereby cured meat colour is formed (Brooks, 1937; Marshall & Marshall, 1945; Koizumi & Brown, 1971; Cheah, 1976; Grozdanov *et al.*, 1976).

Because of the different interpretations regarding the mechanism and kinetics of the cured pigments formation, this process has been examined with a view to resolving the differences.

EXPERIMENTAL

The haemoglobin used in these experiments was purchased from Sigma Chemical Company, Dorset, UK (Hb from sheep erythrocytes, No. H-2750).

Nitric oxide haemoglobin was prepared under anaerobic conditions from a solution of methaemoglobin in 0·2M phosphate buffer, pH 5·5, and nitrite, using ascorbic acid as a reducing agent (molar ratio haem:nitrite:ascorbic acid, 1:10:200, respectively).

The preparation of nitric oxide methaemoglobin was carried out in an atmosphere of nitrogen, by exposing a solution of methaemoglobin (in 0·2M phosphate buffer, pH 6·0) to gaseous nitric oxide.

Nitric oxide was prepared by addition of 0·02M hydrochloric acid to a mixture of sodium nitrite and potassium iodide. Iodine and other impurities were removed by washing the gas with deionised water.

The visible spectra of these derivatives were recorded using a Unicam SP-1800 automatic spectrophotometer and their millimolar extinction coefficients were calculated assuming a molecular weight of 64 500 for sheep haemoglobin.

The concentration of total haem pigments was determined by the cyanmethaemoglobin (CNMetHb) method (Sigma Technical Bulletin No. 525).

The formation of nitric oxide haemoglobin from methaemoglobin and nitrite in the presence of ascorbic acid, was studied within the pH range 4·5 to 6·0, under anaerobic conditions. Specific pH values were maintained by adjusting all reactants to the required pH before mixing, using 0·2M phosphate or acetate buffers.

Reaction mixtures contained 0·0624 mM sheep methaemoglobin,

0·624 mM sodium nitrite and 12·48 mM ascorbic acid. The reactions were run in an anaerobic glass cell at constant temperature (20°C), by first mixing stock solutions of methaemoglobin and sodium nitrite. The cell was then evacuated and the reaction started by addition of ascorbic acid from the side arm.

The conversion was followed spectrophotometrically at 544 nm, the wavelength of maximal absorption of nitric oxide haemoglobin in the visible region.

The reduction of methaemoglobin was investigated within the pH range of 4·5 to 6·2 and was run in air. This resulted in the immediate formation of oxyhaemoglobin (HbO_2) and thus the conversion was followed at 578 nm, one of the absorption maxima of the oxygenated derivative.

Reaction mixtures contained 0·0620 mM sheep methaemoglobin and 12·4 mM ascorbic acid, both in 0·2M phosphate or acetate buffer solutions (molar ratio, haem:ascorbic acid, 1:200).

All experiments were carried out in duplicate using a Unicam SP-1800 spectrophotometer.

RESULTS AND DISCUSSION

The spectra obtained when buffered solutions of sheep methaemoglobin were mixed with sodium nitrite at molar ratios 1:1 to 1:20, haem to nitrite, under either aerobic or anaerobic conditions, are shown in Fig. 1.

Addition of sodium nitrite at the lower molar ratio (1:1, haem to nitrite) did not produce any noticeable change in the spectrum of methaemoglobin. Therefore, spectrum 'a' (shown in Fig. 1) is essentially that of methaemoglobin (extinction coefficients 9·2 and 4·1 mM^{-1} cm^{-1}, at 500 and 630 nm, respectively).

However, a progressive decrease in absorption at both maxima of methaemoglobin was observed with increasing nitrite to haem ratio. The heights of the two shoulders at 535–540 and 565–585 nm were also found to vary with the amount of nitrite, higher absorption values being observed at the higher nitrite concentrations. The spectral changes accompanying nitrite addition showed three isosbestic points at 480, 520 and 610 nm, as shown in Fig. 1.

In order to examine the possibility of nitric oxide pigment formation in the mixtures of methaemoglobin and nitrite, visible spectra were scanned over a period of two or three days of preparation. If the nitric oxide derivative of either the ferric or ferrous pigment had been formed in the mixtures, spectral changes would be expected to occur and to be observed on standing at room temperature and in the presence of oxygen and light,

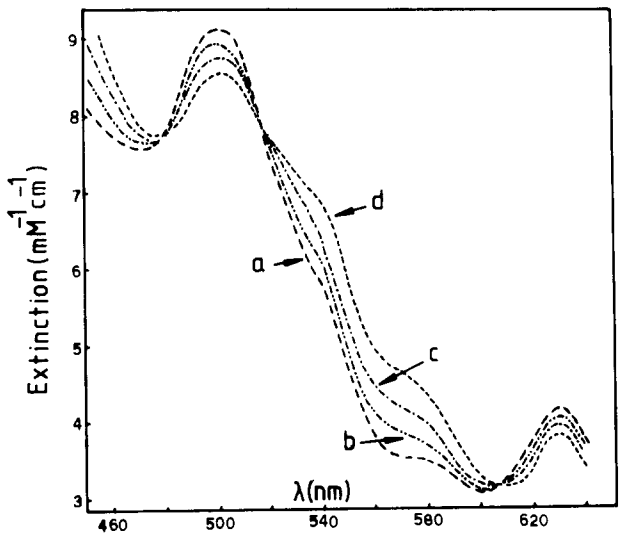


Fig. 1. Spectra of methaemoglobin–nitrite complexes. Molar ratio, haem to nitrite, (a) 1:1, (b) 1:5, (c) 1:10, (d) 1:20.

as a result of the aerobic oxidation of nitric oxide haemoglobin and decomposition of nitric oxide methaemoglobin (Antonini & Brunori, 1971). However, the spectra were found to remain unaltered.

Furthermore, when an attempt was made to extract any nitric oxide pigment present in the preparations by addition of acetone to a final concentration of 80% (Hornsey, 1956), there was a complete precipitation of the haem protein, demonstrating that mixtures of methaemoglobin and nitrite were free of nitric oxide pigment (% extraction 0·30 ± 0·05, at pH range 4·5 to 6·0 and 0·0624 mM haem protein).

All the evidence obtained in the course of this study suggests that, under the experimental conditions applied and the concentrations used, the only product of the reaction between nitrite and sheep methaemoglobin was the ionic complex methaemoglobin–nitrite (MetHb.NO_2), first reported by Barnard (1937), the spectral properties of which were found to be dependent on the actual molar ratio between haem protein and nitrite.

More recently, Uchida & Klapper (1970), presented visible spectra of mixtures of human methaemoglobin and nitrite, very similar to those obtained in this study for sheep methaemoglobin. They attributed the spectra derived from their study to the methaemoglobin–nitrite complexes and suggested that nitrite induces alterations in the molecule of the protein. Metmyoglobin has also been reported to form ionic complexes with nitrite (Fox & Thomson, 1963; Walters *et al.*, 1968).

The increase in absorption at 544 nm after the addition of ascorbic acid

was linear until approximately 50 to 60% of the pigment had been converted to the nitric oxide derivative (Fig. 2).

Below pH 4·5 the end product of the reaction appeared to be a haemochrome (two bands at 534 and 566 nm), probably formed by denaturation of the globin part of the nitric oxide haemoglobin in the acid medium. At the upper limit of the pH range studied, only a small increase in absorption was observed over a period of an hour (Fig. 2).

In order to elucidate the nature of the haemoglobin species involved in the reaction, spectra were scanned at intervals. The spectral changes observed within the pH range of 4·5 to 5·0 were similar, although they occurred at different rates and implied that nitric oxide methaemoglobin was an

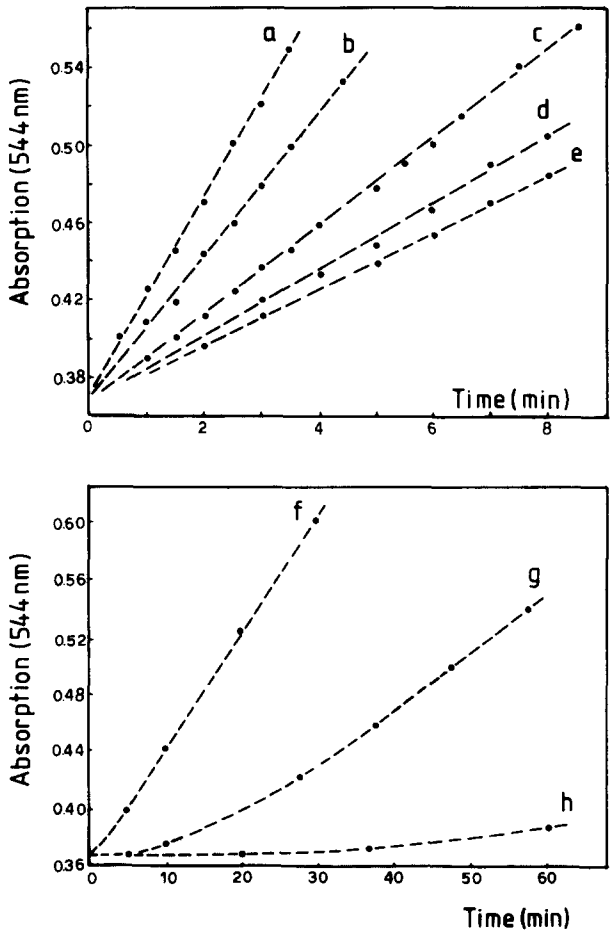


Fig. 2. Absorption changes observed at 544 nm during the course of the formation of nitric oxide haemoglobin. Reaction mixtures pH: (a) 4·55, (b) 4·71, (c) 4·85, (d) 4·95, (e) 5·00, (f) 5·17, (g) 5·50, (h) 5·90.

intermediate in the sequence of reactions whereby nitric oxide haemoglobin was formed from methaemoglobin–nitrite.

Immediately after the addition of ascorbic acid to the reaction mixtures, the absorption at 500 and 630 nm started to drop, while at the same time a small peak appeared at 570–572 nm and a shoulder at 535–540 nm, which eventually developed to another peak at 538–540 nm. During the course of the reaction both peaks shifted toward longer wavelengths until they reached 574 and 544 nm, respectively, i.e. the a and b peaks of nitric oxide haemoglobin (Fig. 3).

Since the absorption maxima of nitric oxide methaemoglobin were found to be at 568 and 535 nm, a slight shift toward shorter wavelengths would be expected if this derivative was formed in appreciable amounts during the course of the reaction.

On the other hand methaemoglobin–nitrite and nitric oxide haemoglobin were found to be isosbestic at 520 nm, whereas nitric oxide methaemoglobin has a higher extinction coefficient at this wavelength (Fig. 4). Thus the small increase of the order of 0·008 absorption units, observed during the initial stages of the reaction at 520 nm, suggested that methaemoglobin–nitrite and nitric oxide haemoglobin were not the only species present in the reaction mixtures and that nitric oxide methaemoglobin was formed during the course of the reaction. As the reaction proceeded, the absorption at 520 nm dropped back to its original value.

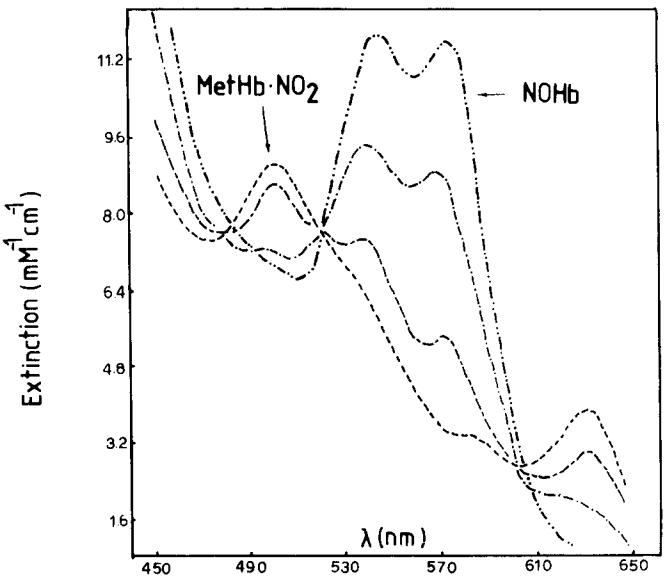


Fig. 3. Spectral changes observed during the course of conversion of methaemoglobin–nitrite to nitric oxide haemoglobin.

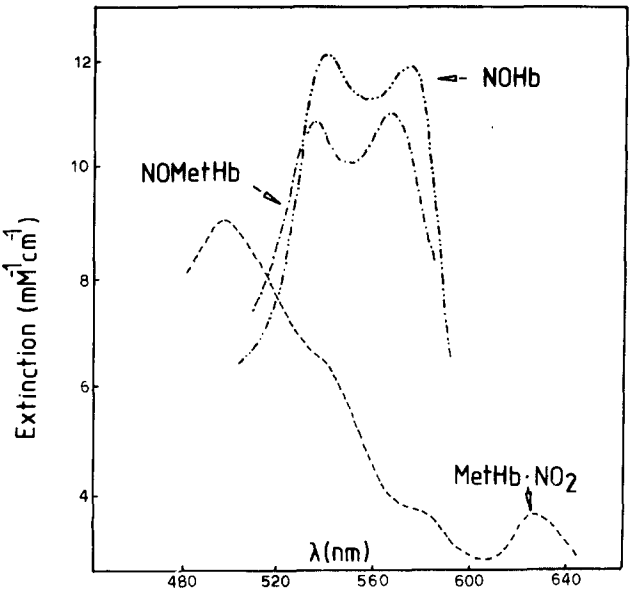


Fig. 4. Superimposed spectra of nitric oxide haemoglobin, nitric oxide methaemoglobin and methaemoglobin–nitrite.

However, at pH values of 5·5 and above, no changes were observed at 520 nm implying that the only detectable species in the reaction mixture were methaemoglobin–nitrite and nitric oxide haemoglobin. Furthermore, the peaks which appeared slowly after the addition of the reductant were those of nitric oxide haemoglobin at 574 and 544 nm.

The spectral changes observed in the pH range 4·5–6·0 gave no evidence to indicate the presence of deoxyhaemoglobin in the reaction mixtures. The visible spectrum of sheep deoxyhaemoglobin is characterised by a single, broad asymmetrical band, with a maximum at 555 nm and a millimolar extinction coefficient of 12·5. Fox & Thomson (1963) and Fox & Ackerman (1968) also failed to detect deoxymyoglobin in their model systems and objected to the postulation of free myoglobin in a system containing nitrite.

The conversion of methaemoglobin–nitrite to the nitric oxide form, as a function of time, was calculated from the changes in absorption at 544 and 520 nm, using the extinction coefficients derived from the spectrophotometric study of the individual haemoglobin derivatives (Table 1) and on the assumption that the only species present during the course of the reaction were methaemoglobin–nitrite, nitric oxide methaemoglobin and nitric oxide haemoglobin (Fig. 5).

The rate of formation of nitric oxide haemoglobin at different pH values (Fig. 6) was derived from the slope of the linear part of the conversion versus

TABLE 1
Extinction Coefficients of Sheep Methaemoglobin–nitrite, Nitric Oxide Methaemoglobin and Nitric Oxide Haemoglobin at 520 and 544 nm

Haemoglobin derivative	E^{520} ($mM^{-1}\,cm^{-1}$)	E^{544} ($mM^{-1}\,cm^{-1}$)
Methaemoglobin nitrite	7·74	5·88
Nitric oxide methaemoglobin	8·50	9·40
Nitric oxide haemoglobin	7·74	12·20

time plots. The same figure shows also the influence of pH on the rate of reduction of methaemoglobin.

Below pH 5·4 the reduction of methaemoglobin is not a step in the sequence of reactions whereby nitric oxide haemoglobin is formed, since the overall reaction cannot be faster than the slowest reaction in the sequence.

Above pH 5·4 the reduction of methaemoglobin was found to be faster than the formation of nitric oxide haemoglobin and therefore is not the rate-limiting step. However, no spectral evidence was obtained to indicate accumulation of the deoxy derivative in the mixtures during the course of the reaction. Ferrous haem pigments are very liable to oxidation by nitrite and therefore, in the absence of a ligand capable of stabilising the ferrous form, any free deoxy haemoglobin would be rapidly reoxidised to the very stable

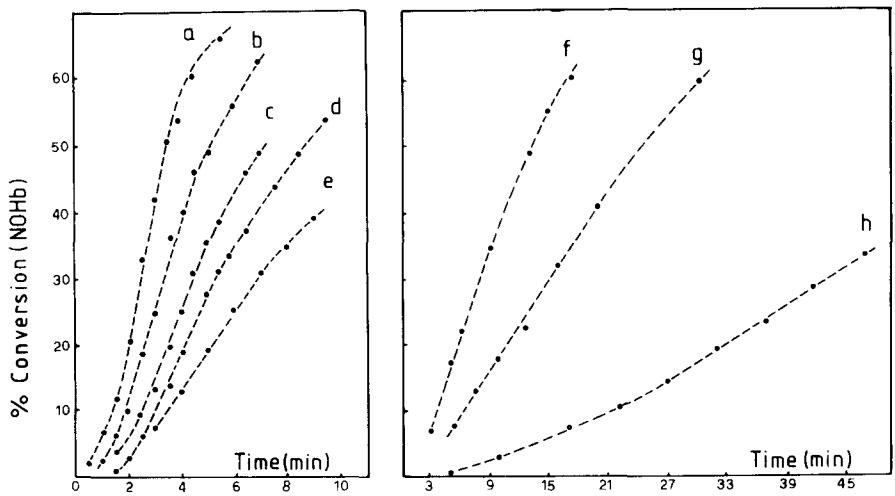


Fig. 5. Time course of the conversion of methaemoglobin–nitrite to nitric oxide haemoglobin. Reaction mixtures pH: (a) 4·55, (b) 4·71, (c) 4·85, (d) 4·95, (e) 5·00, (f) 5·17, (g) 5·50, (h) 5·90.

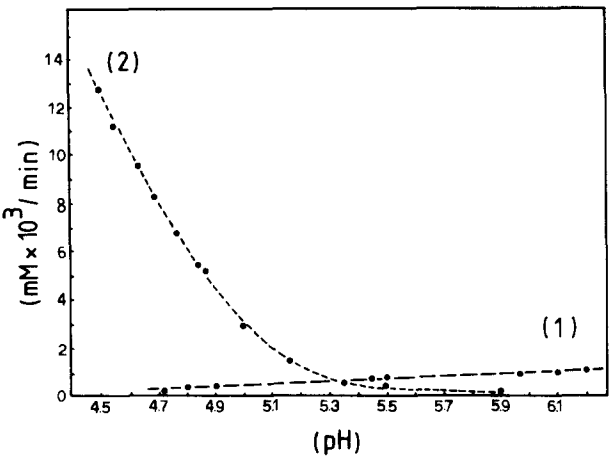


Fig. 6. Influence of pH on the rate of reduction of methaemoglobin (1) and formation of nitric oxide haemoglobin (2).

ferric form. If deoxy haemoglobin, formed from the reduction of methaemoglobin, reacted with nitrite to form equimolecular quantities of methaemoglobin and nitric oxide haemoglobin (Brooks, 1937), the formation of nitric oxide haemoglobin should not have ceased at a pH around 6·0.

It appears therefore that the anaerobic formation of nitric oxide haemoglobin from methaemoglobin, nitrite and ascorbic acid is accomplished through mediation of the unstable derivative nitric oxide methaemoglobin rather than via deoxyhaemoglobin. The fact that nitric oxide methaemoglobin was not detected at pH 5·5 and above, does not necessarily rule out the formation of the intermediate but it rather implies that at the higher pH values, the rate of formation of nitric oxide is slower than the rate of reduction of nitric oxide methaemoglobin and, as a result, the concentration of the latter is kept practically to zero.

Sheep nitric oxide methaemoglobin was found to be unstable under anaerobic conditions and to autoreduce progressively to nitric oxide haemoglobin, even at pH values where the formation of nitric oxide haemoglobin had almost ceased. In the presence of ascorbic acid, the reduction of the intermediate must therefore be accomplished readily. Since there was no indication that methaemoglobin could reduce nitrite directly, the formation of the ligand (nitric oxide or nitrosyl group) must be carried out exclusively by the reductant and appears to be the rate-limiting step in the sequence.

The sharp decrease in the rate of formation of nitric oxide haemoglobin with increasing pH suggests that it is the undissociated forms of nitrous and ascorbic acid which are involved in the formation of nitric oxide. According

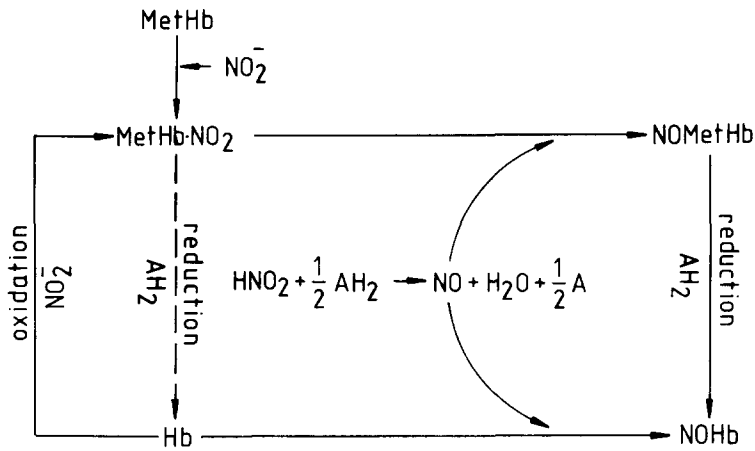
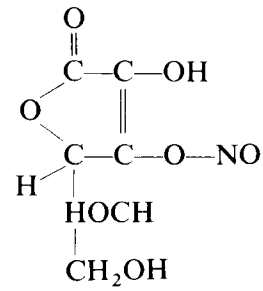


Fig. 7. Suggested mechanism for the anaerobic formation of nitric oxide haemoglobin from methaemoglobin–nitrite and ascorbic acid.

to Fox & Thomson (1963), in the presence of both nitrite and ascorbic acid (AH_2), a semistable nitric oxide–ascorbic acid intermediate is formed (AHNO), which may either undergo a bimolecular reaction in the backward direction to reform the initial reactants, or react to release nitric oxide:

$$HNO_2 + AH_2 \rightarrow AHNO + H_2O \text{ (fast)}$$
$$2\,AHNO + H_2O \rightarrow 2\,AH_2 + N_2O_3 \text{ (fast)}$$
$$AHNO \rightarrow AH + NO \text{ (slow)}$$

The last reaction, being the slowest in the sequence, is considered to be the rate-limiting step. Direct evidence for the existence of this intermediate has not been obtained, but may be represented as:



Viewed overall, a mechanism which would take into account the observations from this present study and other published works, is that shown in Fig. 7. The reaction represented by the broken arrowed line is unlikely to be a prominent feature for reasons already considered.

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