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## Oxygen Bonding to Haemoglobin. $^{17}\text{O}$ NMR Spectrum — A Second Look\*

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It was found that the upper concentration limit in detecting the oxygen-17 NMR signal from ordinary solvent water in solutions of oxyhaemoglobin is about  $12 \cdot 10^{-3}$  M (in haem). From samples of oxyhaemoglobin prepared with oxygen gas enriched to 62% in  $^{17}\text{O}$  a signal could be detected within a few days in spite of higher protein concentrations. Such signals increased in couple of weeks up to three times. However, within this period of time the sealed, dissolved oxyhaemoglobin became deoxygenated showing the characteristic colour change, while the NMR  $^{17}\text{O}$  signal persisted. The pressure above such sealed solutions diminished and the mass-spectroscopic analyses showed two- to threefold enrichment in  $^{17}\text{O}$  of solvent water after the deoxy-conversion, while in the gas phase oxygen was replaced by carbon dioxide. There is no deoxy-conversion in samples prepared under sterile conditions or in presence of sodium azide (equimolar with haem). It is concluded that owing to bacterial contamination in haemoglobin solutions prepared under ordinary conditions oxygen from the gas-phase is being reduced into water through bacterial metabolism. Thus, the observed  $^{17}\text{O}$  NMR signals were due to the solvent water enriched in  $^{17}\text{O}$ . A former paper\* claiming detection of  $^{17}\text{O}$  NMR signal from oxygen bound to haemoglobin is thus invalidated.

### INTRODUCTION

The rationale of oxygenating haemoglobin solutions with oxygen gas enriched to 65% in  $^{17}\text{O}$  and recording the NMR spectra was explained in Paper I. It was hoped to obtain information on the mode of oxygen bonding to haemoglobin<sup>1</sup> (see also here the consecutive paper by Velenik and Lynden-Bell). The coincidence of the recorded line with that of  $^{17}\text{O}$  NMR from water

\* S. M. reported on the subject of this paper at the conference *Magnetic Resonances in Biological Research* on 14th September 1969 in Cagliari, Italy. — The original paper, which we shall be referring hereinafter to as Paper I, is by S. Maričić, J. Leigh Jr., and D. Sunko, *Nature* **214** (1967) 462.

asked for more experiments, in the course of which we eventually came to the conclusion that what we have been observing all the time has been indeed a spectrum originating in  $^{17}\text{O}$  of water enriched by reduction of  $^{17}\text{O}$  from the gas phase. This report discusses the NMR and other experimental evidence together with some efforts at explaining this transfer of oxygen from the gas-phase into the solvent (water).

#### EXPERIMENTAL

##### *Sample Preparation*

Except in the sterile preparation, bovine haemoglobin was used throughout this work. The red cells were washed five times with 0.9% saline centrifuging for 10 min each time at successively higher speeds. The washed red cells were then left overnight to freeze at about  $-10^{\circ}\text{C}$ . After thawing next day phosphate buffer (0.1 M  $\text{KH}_2\text{PO}_4$ , pH 7) was added to the red cells and stroma was centrifuged off for 30 minutes at 13000 g. The clear supernatant was then subjected to extensive dialysis against the phosphate buffer till negative reaction to chloride ions. Whenever possible most of the steps of the procedure were performed close to  $4^{\circ}\text{C}$ .

In order to get better purification in some samples crystallization was included before the final dialysis. The procedure described by Roche *et al.*<sup>2</sup> for bovine haemoglobin was used. The crystals were dissolved in the phosphate buffer and dialysed as usual.

Semisterile conditions were maintained later on. The whole technique was as described except for using the sterilized glassware and taking care to avoid oral infection. In some of the preparations antibiotics were added: 200000 I. U. of penicilline G and 0.25 g. of dihydrostreptomycin («Pliva» — Zagreb) per liter of any of the solutions used in the course of preparation.

For concentrating the dialysed solutions mostly polyethylene-glykol M. W. 17000—20000,  $n = 400-450$  («Carbowax» — Serva, Heidelberg) was used, but in a few preparations it was replaced by glucose.

The whole preparative procedure till the final concentrated solution of haemoglobin took usually about a week, and the experiments started mostly within the following week.

The sterile haemoglobin was prepared from freshly drawn human blood. There was a slight difference in the procedure in that after washing the erythrocytes three times with 0.9% saline on the centrifuge, they were haemolyzed by sudden hypotony (*i. e.* adding a tenfold volume of distilled water), then adjusted to pH 5 with acetic acid. Afterwards, the stroma was removed by centrifugation, and the solution adjusted to 0.1 M potassium phosphate, pH 7.0, during the sterile filtration and concentration.<sup>3</sup>

For work with oxygen isotopes the ampoules filled with Hb-solutions were sealed onto the vacuum line. In order to suppress foaming during the degassing stage they were initially flushed several times with pure helium (Air-Products, Inc., U.S.A.). The oxygen isotope was then introduced by Toepler pump. The ampoules were provided with break-seals.

The concentrations (in mM per haem) were determined by spectrophotometry either from the spectrum of oxyhaemoglobin ( $E_{415} = 127$ ,  $E_{578} = 15.6$ ,  $E_{542} = 14.7$ ), or by the cyanmet-method<sup>4</sup> and iron determination.<sup>5</sup>

The bacteriological control consisted of the usual incubation of 0.1 ml sample (properly diluted) onto Oxo agar for 24 hours. The results were expressed as number of developed colonies per 1 ml of sample. Normally the incubation was at  $37^{\circ}\text{C}$  but in order to check on the viability of the bacteria controls were incubated at  $4^{\circ}\text{C}$  and it was found that for 3—4 days there was no development of bacteria. They started to grow afterwards in a more or less normal way. The ratio of optical densities at 280 and 415 nm was used as an additional control of bacterial growth.

### The Chemicals

Oxygen-16 was used from ordinary gas cylinders without any purification.

Oxygen-17 and oxygen-18 was produce of Yeda, Rehovoth, purchased through Miles, Ltd. with the following isotopic analyses: Oxygen-17: 61–62%  $^{17}\text{O}$ ; 24.4%  $^{16}\text{O}$  14.4%  $^{18}\text{O}$  (by mass-spectroscopy of refilled ampoules). Oxygen-18: 0.425%  $^{17}\text{O}$ ; 95.6%  $^{18}\text{O}$  (as given by the producer).

All the other chemicals were of analytical grade purchased from various sources.

### NMR Measurements

All the measurements but one were done on a home made spectrometer comprising of a permanent magnet,<sup>6</sup> and a modified Robinson-type<sup>7,8</sup> NMR detector for 7.33 MHz. The NMR detector comprises a noise optimised RF oscillator and LF detector and works up to 1.3 Vrms RF signal across the input tank circuit. To improve the frequency stability of the RF oscillator a special control feed-back loop was added to the NMR detector. Its main feature is that it can be used both for a stabilization of the frequency of the RF oscillator (magnetic sweep for taking NMR spectra) and for the linear frequency sweep of the same oscillator (using a ramp voltage generator). The low frequency NMR signal from the output of the NMR detector is led to the PAR lock-in amplifier and then to a recorder. The measurements were performed with the modulation frequency of 80 Hz, a time constant at the output of the synchronous detector of 20 s, and magnetic sweep rate of 0.15 G/min, at about 0.2 G of the  $^{17}\text{O}$  NMR line width in pure water.

The RF power was optimized and it was found as before (Paper I) that saturation takes place at about twice its value for the haemoglobin solutions as compared to that required for ordinary water.

The modulation amplitude had to be about half the line width. The line-shapes are not corrected for any of the instrumental broadenings.

3 ml sample of ordinary water (same sample size as in haemoglobin measurements) was used throughout for checking the spectrometer performance while a sample of 0.5 ml 11%  $^{17}\text{O}$  water served only for aligning the magnetic field, whose homogeneity was better than 1:10<sup>6</sup> over the sample volume.

### Mass-spectrometry

The isotopic analyses were carried out with a Nier-type mass-spectrometer designed for determinations of small isotopic variations.<sup>9</sup> The gas-phase above Hb-solutions was recuperated on freezing the solutions, into the vials for mass-spectrometry, through a Toepler-pump. The solvent water was redistilled under *vacuo*. These samples were then used for mass-spectrometry without any further purification. Oxygen-17 and oxygen-18 were measured on oxygen and carbon dioxide. The samples of water were exchanged with potassium bromate at 40° C.<sup>10</sup> A drop of hydrochloric acid was added to catalyze the exchange rate. Because of small samples water was redistilled *in vacuo* from the bromate solution. Dried potassium bromate was converted to O<sub>2</sub> for the massspectrometric analysis by thermal decomposition. Measurements of 33/32 and 34/32 ratios were used for  $^{17}\text{O}$  and  $^{18}\text{O}$  respectively, for oxygen samples. In case of carbon dioxide the content of the two oxygen isotopes was derived from the ratios 45/44 and 46/44 respectively. The correction for isotopic dilution of enriched water samples by potassium bromate was taken into account.

## RESULTS AND DISCUSSION

### 1) The line-widths of oxygen-17 NMR spectra from water in concentrated haemoglobin solutions

Our line-widths measurements of  $^{17}\text{O}$  lines on CO-haemoglobin solutions (within another independent line of study) showed that in solutions of about 12 mM the line becomes hardly observable due to broadening caused by interaction of water molecules with the protein surface (*i.e.* increase of correlation time) and their fast exchange with the bulk solvent.<sup>11</sup>

TABLE I  
 $^{17}\text{O}$ -NMR-Line Widths from Solvent Water  
of Haemoglobin Solutions under  $^{17}\text{O}_2$ -Gas  
(at  $25^\circ \pm 1^\circ \text{C}$ )

Hb-concentration [mM]	21.8	17.6	15.8	15.0	14.0
Line width* [mG]	552	467 $\pm$ 20	417 $\pm$ 6	400 $\pm$ 20	384 $\pm$ 10

\* uncorrected for instrumental broadening, but all measured under the same conditions.

Table I summarizes similar data from our measurements on oxy-17-haemoglobin solutions *that have been in contact with the  $^{17}\text{O}$  gas-phase for several days*. This data extends the straight-line dependence of line-widths on protein concentration above that obtained with CO-haemoglobin solutions, *i. e.* with natural abundance of  $^{17}\text{O}$  in ordinary water.

The conclusion was that whatever the origin of the NMR line in the first experiment (Paper I) or, for that matter, in those that followed it, the line cannot be ascribed only to the signal from  $^{17}\text{O}$  contained in the *initial* ordinary solvent-water, because it could not be detected at the high protein concentrations used.

## 2) Oxy $\rightarrow$ deoxyhaemoglobin conversion

Our samples of haemoglobin were sealed off the vacuum line under a pressure of oxygen (containing 62%  $^{17}\text{O}$ ) of approximately 300 mm Hg so that the haemoglobin was fully oxygenated, as verified by handspectroscope. In spite of such a closed system supposedly at equilibrium, we found that on standing it turned into deoxyhaemoglobin, as revealed also by handspectroscope. The final pressure above the samples decreased substantially from its original value. This led to the isotopic examination of the liquid phase. The mass-spectroscopic results on  $^{17}\text{O}$  and  $^{18}\text{O}$  abundance in water from haemoglobin solutions after the oxy  $\rightarrow$  deoxy conversion (Table II) indicate more than a twofold enrichment.

TABLE II  
Mass-spectroscopic Data on the  $^{17}\text{O}$  and  $^{18}\text{O}$  Abundance in Water from  
Haemoglobin Solutions after the Oxy  $\rightarrow$  Deoxy Conversion

Initial gas	Hb-conc. mM in haem	Weeks after oxygenation	Isotopic abundance atom %	
			$^{17}\text{O}$	$^{18}\text{O}$
control measurements of plain ordinary water			0.037	0.211
			0.041	0.212
(62%) $^{17}\text{O}$	15.8	12	0.060	0.211
	14.1*	7	0.151	0.24
(95%) $^{18}\text{O}$	15.0*	4		0.40
	15.0*	6		0.53

\* gas-phase data in Table III.

Further, a semiquantitative evaluation of the signal intensities (by taking the products of the line-widths and peak-to-peak amplitude) showed a two- to threefold increase of the signals of each sample within a week or two (for illustration, see Fig. 1.).

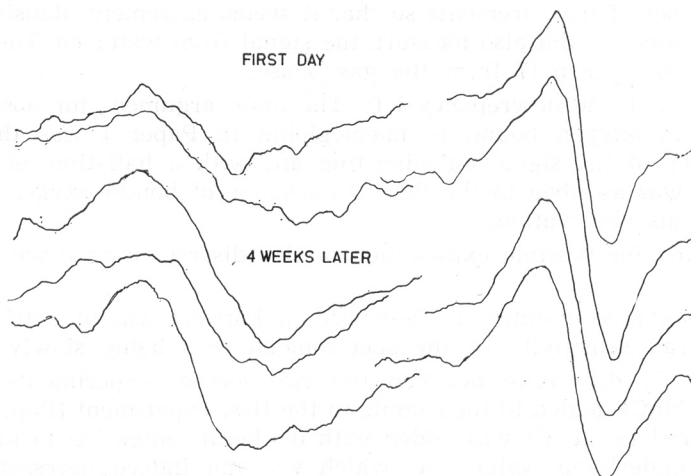


Fig. 1.  $^{17}\text{O}$ -NMR line shapes from solvent water of a 14.0 mM solution of haemoglobin (3 ml) after it has been 1 day (upper curves, left) and over 4 weeks (lower curves, left) under an initially 62%  $^{17}\text{O}$  oxygen. For comparison,  $^{17}\text{O}$  spectra of 3 ml ordinary water (taken alternately with Hb spectra) are given on the right.

Merely from the standpoint of the NMR experiment the last results cast serious doubt as to the validity of our first spectrum (Paper I), because we now observe the same  $^{17}\text{O}$  line both in the presence of oxy- and deoxyhaemoglobin.

We checked on this point in two experiments. First, a sample was degassed ( $^{17}\text{O}$  recuperated) on the vacuum line after it had undergone the oxy $\rightarrow$ deoxy conversion. The  $^{17}\text{O}$  line was nevertheless recorded, just as it was before degassing, with deoxyhaemoglobin in solution.

Second, with another sample this sequence of results was obtained: a 15 mM deoxygenated sample showed no NMR  $^{17}\text{O}$  signal, as expected; on oxygenation with about 360 mm Hg of  $^{17}\text{O}$  a signal appeared within 24 hours; the signal increased during the next two days, while the sample remained oxyhaemoglobin; the gas phase of this sample was then expanded into a 100-fold pre-evacuated volume and after ascertaining that the sample turned into deoxyhaemoglobin it was sealed off. The NMR signal was observed afterwards, as it was seven weeks later and also again five months since the deoxygenation.

The ampoule was then opened and air readmitted, thus reoxygenating (with  $^{16}\text{O}$ ) the sample and the signal was being recorded, but it neither disappeared nor showed any sign of diminishing intensity within 48 hours.\*

\* We are very much indebted to Dr. Jay A. Glasel of Columbia University for making available to one of us (M.P.) the Varian DA-60 spectrometer for the last two control NMR-recordings.

### 3) *Reevaluation of the first experiment (Paper I)*

As described in Paper I our first sample of  $^{17}\text{O}$  oxygenated haemoglobin was used in NMR measurements 11 months after it was oxygenated.

The position, width, saturation characteristics, all agree between the first and present set of measurements so that it seems extremely plausible that in fact at that time we did also measure the signal from water enriched through a reduction of oxygen-17 from the gas phase.

There is but one discrepancy left. The main argument for observing the  $^{17}\text{O}$  line from oxygen bound to haemoglobin in Paper I was the gradual disappearance of the signal on admitting air, with a half-time of about six hours. This was ascribed to the  $^{17}\text{O}/^{16}\text{O}$  exchange of bound oxygen. We could not repeat this observation.

In looking for possible explanation of this discrepancy we see two possibilities:

a) the decrease in intensity described in Paper I was of a trivial cause, i.e. the overall sensitivity of the spectrometer was being slowly degraded;

b) the only difference between the two sets of experiments is in the presence of  $\text{MnCl}_2$  added to the sample in the first experiment (Paper I) before it was exposed to air. (It was added with deliberate intention to broaden the supposedly underlying water line, which was not indeed necessary because that line is anyway broadened beyond detectibility by high protein concentration.) One of the referees of Paper I doubted the validity of our argument by supposing that the line was only due to  $^{17}\text{O}$  from water but was slowly broadened by  $\text{MnCl}_2$ . Against this is our observation (Paper I) of instantaneous broadening on adding  $\text{MnCl}_2$ , of the  $^{17}\text{O}$  NMR water line in ordinary solution of Hb, though in a less concentrated solution (7 mM), so that this point ought to be checked yet. It is more important to know what is the origin of the observed oxy $\rightarrow$ deoxy conversion and could it be circumvented in order to be able to proceed safely with NMR experiments.

### 4) *Origin of oxy $\rightarrow$ deoxy conversion*

In the early stage of these experiments our attention was drawn to the possibility that bacterial metabolism in our samples may cause the observed deoxygenation.\*

With this in mind, we looked into the composition of the gas phase after the discussed conversion had taken place. Table III comprises the results obtained by mass spectrometry. It seems beyond doubt that not only the main part of oxygen from the initial gas phase was reduced to water as evidenced by the results in Table II, but that this process was accompanied by  $\text{CO}_2$  evolution which practically replaced oxygen from the gas phase at the end of experiment. This, of course, explains the deoxygenation of the samples.

Although the replacement of oxygen by carbon dioxide strongly supports the bacterial hypothesis it is still not a direct proof for it. In fact we ask whether haemoglobin itself participates in the reductive mechanism by which oxygen is being transferred into water?

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\* We should like to thank very much Dr. Joan Keilin of Cambridge University for this suggestion.

TABLE III

*Mass-spectroscopic Data on the Composition of the Gas-phase after the Oxy → Deoxy Conversion*

Initial gas approx. at 0.5 at	Hb-conc. mM in haem	Weeks after oxygenation	Composition vol. %				CO <sub>2</sub> isotopic analysis atom %		
			H <sub>2</sub> O	N <sub>2</sub>	O <sub>2</sub>	CO <sub>2</sub>	<sup>16</sup> O	<sup>17</sup> O	<sup>18</sup> O
62 <sup>0</sup> / <sub>0</sub> <sup>17</sup> O	19.0	2	0.7	1.5	56.5	41.2	96.6	2.46	0.94
	17.6	2	0.2	—	2.3	97.5	99.5	0.29	0.25
	21.8	14	0.7	5.0	1.3	93.0	99.4	0.33	0.27
	14.0	7*	0.7	4.2	—	95.0	99.3	0.38	0.29
95 <sup>0</sup> / <sub>0</sub> <sup>18</sup> O	15.0	4*				>99			4.35
		6*				>99			5.15

\* liquid phase data in Table II.

Haemoglobin proper does not take part in this process. The direct proof came from an experiment with sterile sample of human haemoglobin, 16 mM in haem, sealed under air.

After five months there was no deoxygenation and in two of the vials the composition of the gas phase (after two months) was as given in Table IV, which also indicates that no change has taken place in this closed system with sterile haemoglobin.

TABLE IV

*The Composition of the Gas-phase*  
Initially air, after 8 weeks in contact with sterile 16 mM oxy-16-haemoglobin solution.

	vol.—%	
	sample 1	sample 2
CO <sub>2</sub>	1.1	0.7
A	0.7	0.8
O <sub>2</sub>	25.0	22.5
N <sub>2</sub>	73.0	76.0

There was no deoxy-conversion also in ordinary samples sealed or closed under air in presence of sodium azide in a concentration greater than 1:1 on a molar basis referring to haem. It is known that azide ion does not change the spectrum of neither oxy-, nor deoxy-haemoglobin as distinct from ferri-haemoglobin.<sup>12</sup> Nevertheless, in view of unusually high concentrations of our samples, we checked on this and found agreement. The spectrophotometric ampoules were closed air-tight and haemoglobin remained in its oxyform for more than two months. The azide experiment excludes haemoglobin only as a reductive agent. Both bacterial metabolism and most of other protein components with kind of catalase activity may equally well be poisoned by

the azide ion. However, the addition of azide for preserving oxyhaemoglobin in NMR work is very practical because it eliminates special sterile techniques.

As already concluded, the sterile and the azide experiment definitely exclude haemoglobin itself as a reductive agent. The sterile experiment also indicates that bacteria may cause the whole trouble in normally prepared haemoglobin solution, but, together with the azide experiment some doubt is still left as to whether there is yet another unknown enzyme component which may act reductively. Namely, the difference in haemolysis of erythrocytes (hypotony with acidification *vs.* freezing without hypotony) in the sterile *vs.* other preparations may have caused removal of any such catalytic substances in the sterile as distinct from other experiments.

On balance we believe that bacterial metabolism did indeed transfer oxygen into the liquid phase of our samples, but we cannot completely reconcile this conclusion with the kinetics of pressure-change above the closed Hb-solutions.

Also, the conversion works at 4° C without any dramatic difference compared to room temperature experiments, although the standard bacteriological control at 4° C was usually negative. The bacterial contamination in our experiments varied between 10<sup>5</sup> per ml of original solution and a few tens, at the beginning, increasing by a factor of 100 to 10 at the end. The ratio of O.D.<sub>280</sub>/O.D.<sub>415</sub> was practically the same before and after experiments. Rare cases of differences in this ratio amounted to some 10% around a value of 0.3. Bacteriograms indicated external contaminations with staphylococcus and pseudomonas as well as endogenous by achromobacteria. The former used to develop easily countable point colonies, while the latter appeared on incubation more like a fuzzy homogeneous veil, and were most resistant, staying even after severe thermal shock treatment (tenfold freezing in liquid air with subsequent immediate thawing in water at 37° C). Besides, haemoglobin itself does not seem to enable true logarithmic phase growth of bacteria. They rather seem to multiply at their own expense in the presence of oxygen. All this calls for great care in defining the purity of such very concentrated haemoglobin samples.

As for recording <sup>17</sup>O spectrum of oxygen bound to haemoglobin, the main outcome of the present report, apart from invalidating the first experiment (Paper I), is that it seems indeed almost hopeless to look for the signal in this classical way.

At the moment, having found the way of stabilizing the oxyhaemoglobin samples during long periods of time, the main problem of sensitivity remains, because in all probability the NMR line width of <sup>17</sup>O bound to haemoglobin will be wider than the H<sub>2</sub>O—<sup>17</sup>O-line broadened by high protein concentration.

At least there is a guide now in searching for the <sup>17</sup>O NMR signal from oxygen bound to haemoglobin as described in the following paper by A. Velenik and R. Lynden-Bell<sup>13</sup>.

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## IZVOD

## Vezivanje oksigena na hemoglobin. Spektar NMR oksigena-17 — revizija

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Nađeno je da je gornja granica koncentracije u određivanju signala NMR oksigena-17 vode kao otapala u otopinama oksihemoglobina oko  $10 \cdot 10^{-3}$  M (po hemu). Usprkos visokim koncentracijama proteina, signal oksigena-17 pojavljuje se unutar nekoliko dana kod uzoraka oksihemoglobina pripremljenih s obogaćenim plinom oksigena (62% na<sup>17</sup>O). Ti signali porastu do tri puta unutar nekoliko tjedana. Međutim, u isto vrijeme otopina oksihemoglobina se u zataljenoj ampuli deoksigenira pokazujući karakterističnu promjenu boje, dok se signal NMR oksigena-17 ne gubi. Maseno-spektroskopska analiza pokazuje obogaćenje vode na <sup>17</sup>O dva do tri puta nakon deoksigenacije, dok se u plinskoj fazi oksigen zamjenjuje s karbon-dioksidom.

U uzorcima hemoglobina, pripremljenim pod sterilnim okolnostima ili uz prisutnost natrium-azida, ne dolazi do promjene oksihemoglobina u deoksihemoglobin. Može se zaključiti da je zbog bakterijske kontaminacije otopina hemoglobina, pripremljenih na uobičajeni način, došlo do redukcije oksigena iz plinske faze u otapalo — vodu, putem bakterijskog metabolizma.

Prema tome, nađeni signal NMR oksigena-17 potječe od vode-otapala obogaćene na tom izotopu, te se ne može pripisati oksihemoglobinu, kao što je to učinjeno u ranijem radu.

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