

## The Simultaneous Determination of Bis(hydroxymethyl)-peroxide (BHMP), Hydroxymethylhydroperoxide (HMP), and H<sub>2</sub>O<sub>2</sub> with Titanium(IV). Equilibria Between the Peroxides and the Stabilities of HMP and BHMP at Physiological Conditions

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1. A method is described for the assay of aqueous solutions of bis(hydroxymethyl)peroxide (BHMP), hydroxymethylhydroperoxide (HMP), and H<sub>2</sub>O<sub>2</sub>, singly or in mixtures. It is based on the different reactivities of the peroxides with Ti(IV) in dilute sulfuric acid.

2. The NMR spectra of BHMP and HMP are presented.

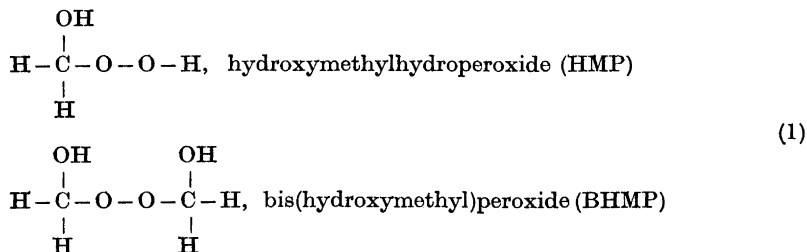
3. The hydrolyses of BHMP to HMP and HCHO, and of HMP to H<sub>2</sub>O<sub>2</sub> and HCHO are acid and base catalyzed. BHMP is rapidly hydrolyzed at physiological pH, *t*<sub>1/2</sub> being around 30 sec at pH 7 and room temperature. Biochemical effects, hitherto attributed to this peroxide, are hence possibly caused by HMP.

4. The rates of formation of BHMP from HMP and HCHO, and of HMP from H<sub>2</sub>O<sub>2</sub> and HCHO are presented.

5. The equilibrium constants between BHMP, HMP, H<sub>2</sub>O<sub>2</sub>, HCHO and H<sub>2</sub>O are determined.

6. The activation energy of the hydroxyl ion catalyzed hydrolysis of BHMP is 39 kJ mol<sup>-1</sup> within the range 0–35°C.

When formaldehyde and hydrogen peroxide are mixed, a number of organic peroxides are formed. The simplest, hydroxymethylhydroperoxide, and bis(hydroxy)methylperoxide, contain one –O–O–, but by substituting perhydroxy groups for hydroxyl groups several more may be formed.<sup>1</sup>

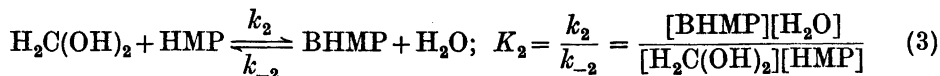
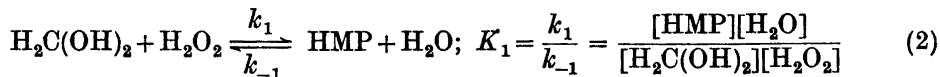


The mutagenic effects of BHMP<sup>2,3</sup> and its inhibitory action on peroxidase,<sup>4</sup> blood catalase,<sup>5</sup> anaerobic glycolysis, cell respiration, Ehrlich-ascites-carcinoma cells *in vitro*, aldolase, glyceraldehyde-3P-dehydrogenase, and lactate dehydrogenase<sup>6,7</sup> have been studied. In addition a good effect on mouse ascites-carcinoma *in vivo* has been reported.<sup>8</sup> Hilz and Eckstein<sup>9</sup> described the action of BHMP on the cell-division, glycolysis and cell respiration of synchronized yeast cells.

However, the stability and behaviour of BHMP under physiological conditions is incompletely known, and it therefore cannot be taken for granted that the effects ascribed to BHMP in the above papers are a direct result of this compound.

Wieland and Wingler<sup>10</sup> who described BHMP synthesis, studied its reactions in alkaline water solution and at high temperature in neutral and acid solutions. Jaillet and Oullet<sup>11</sup> investigated the reactions of BHMP in alkaline solution. When determining the freezing points of mixtures of HCHO and H<sub>2</sub>O<sub>2</sub>, they got depressions which were interpreted as caused by equilibria between HMP, BHMP, and BHMP(HCHO)<sub>2</sub>. Jenkins and Style<sup>12</sup> presented thermochemical data on BHMP. These authors also investigated the thermal decomposition of BHMP in the gas phase at  $t > 110^\circ\text{C}$ . By means of elementary analysis and peroxide determination, Serdaroglu<sup>13</sup> claimed to have demonstrated that BHMP crystallizes with 0.5 moles of water. Rieche and Meister assumed an equilibrium between H<sub>2</sub>O<sub>2</sub>, HMP, and BHMP, and claimed the preparation of pure HMP under anhydrous conditions.<sup>14,15</sup> Equilibrium constants have been determined at low temperature (freezing point depression)<sup>16</sup> and at room temperature in acid solution with an admittedly inadequate method.<sup>17</sup>

In the present investigation, equilibria between H<sub>2</sub>O, (hydrated) HCHO, H<sub>2</sub>O<sub>2</sub>, HMP, and BHMP were presumed to exist; they are defined as



The experiments were performed in dilute water solutions. The equilibrium constants and two of the rate constants will therefore be presented with the concentration of water (55.6 M) included;

$$K_1' = K_1[\text{H}_2\text{O}]^{-1}; \quad K_2' = K_2[\text{H}_2\text{O}]^{-1} \quad (4)$$

$$k_{-1}' = k_{-1}[\text{H}_2\text{O}] \quad \text{and} \quad k_{-2}' = k_{-2}[\text{H}_2\text{O}]$$

This paper presents methods for the quantitation of the above compounds, based on the different reactivities of the peroxides with Ti(IV) in an acid aqueous medium. The NMR spectra of HMP and BHMP are presented. The equilibrium constants  $K_1'$  and  $K_2'$  (eqn. 4) and the rates of formation of HMP and BHMP have been determined. The stabilities of the two organic peroxides, their acid and base catalyzed hydrolysis, and the energy of activation of the latter reaction have also been studied.

## MATERIALS AND METHODS

Unless otherwise stated, the experiments were performed at 25°C.  $H_2O_2$  (30 w/w) *p.a.*, Perhydrol Merck.  $[H_2O_2]$  was determined by titration with  $KMnO_4$ , standardized with  $Na_2C_2O_4$ .<sup>18</sup> Formaldehydlösung (35 % w/w) *p.a.*, Merck.  $[HCHO]$  was assayed with sulphite.<sup>19</sup> Ether, Mallinckrodt, *a.g.* Only freshly opened tins were used.  $TiOSO_4$ , *p.a.*, Riedel-de-Haen AG. Acetic acid, *p.a.*, Merck. Cacodylic acid, 99.5–100.5 %. B.D.H. Tris, Sigma, *a.g.*  $H_2O$ , double distilled from quartz vessels.

NMR spectra were recorded at 0°C on a Varian A-60 A instrument at 60 MHz. The peroxides were dissolved in hexa-deuterio-acetone to give a concentration of about 10 %. Corrections were made for solvent protons. All shifts are expressed in  $\delta$ -values (ppm) with reference to TMS as internal standard. For spectrophotometry a Beckman DK2-A with its cuvette house and holder thermostated at 25°C was used. The pH-values were determined with a Radiometer pH-meter 25 (Copenhagen), calibrated at 25°C against  $KH_2(C_2O_4)_2$ , potassium biphthalate and phosphate. For other temperatures the pH-values of the reference solutions were corrected according to Bates.<sup>20</sup>

*Synthesis of BHMP.*  $H_2O_2$  (30 %) and HCHO (35 %) were mixed in the molar proportions 1:2 in a petri dish, which was placed in a desiccator over  $P_2O_5$  in vacuum. After a day or two needle-shaped crystals were formed which were washed with a little ether and recrystallized from this solvent. Yield  $\geq 70$  %. Melting point 63–64°C (Kofler, uncorr.). Assay for total peroxide according to the method described below, gave  $\sim 99$  % of the theoretical value.

*Synthesis of HMP.* HMP has only been obtained in an equilibrium mixture with BHMP and  $H_2O_2$ , never alone. Equimolar amounts of HCHO (35 %) and  $H_2O_2$  (30 %) were mixed in a petri dish and placed in a desiccator over  $P_2O_5$  in vacuum. The resulting oil is volatile and must be kept at  $-20^\circ C$ . The synthesis can also be performed in diethylether. HCHO (35 %) and  $H_2O_2$  (30 %) were shaken separately with  $3 \times 1$  vol. of ether for 30 min, the extracts dried for two days with water-free  $Na_2SO_4$  at 0°C,<sup>1</sup> and the concentrations of  $H_2O_2$  and HCHO assayed (around 3.5 %). Equimolar amounts of HCHO and  $H_2O_2$  were mixed and left for 1 day in a desiccator, whereafter the ether was distilled off.

In one experiment HCHO and  $H_2O_2$  were mixed in different proportions, deviating slightly from a 1:1 ratio and the compositions of the oils were determined (Table 1).

Table 1. The composition of the oily product ("HMP") as a function of the molar proportions of  $H_2O_2$  to HCHO. The synthesis was performed in ether as described in the text.

$H_2O_2/HCHO$	$H_2O_2$ %	HMP %	BHMP % (of total peroxide in the batch)
1.05	17.4	68.9	13.7
1.0	14.5	71.6	14.0
0.95	12.0	71.5	16.5
0.91	9.9	71.4	18.7

### Methods for the determination of organic peroxides

Peroxides were analyzed by means of a colour reaction with Ti(IV) and by NMR. The procedure for determining  $H_2O_2$ , HMP, and BHMP by the first method is described in detail below. When applicable, NMR gave results in good agreement with those of the first method.

Ti(IV) forms a 1:1 compound with  $H_2O_2$  in acid aqueous medium.<sup>21</sup> The compound is bright yellow and has a flat absorbance maximum around 405 nm. A yellow colour appears with HMP and BHMP too, though at slower rates. The methods for the simulta-

neous determination of  $H_2O_2$ , HMP, and BHMP presented here, are based on the different reactivities of the peroxides with Ti(IV) in acid aqueous medium.

*The Ti stock reagent* (modified from from Ref. 22). 6.6 g  $TiOSO_4$  were ground and placed into 50 ml  $H_2O$  and 20 g  $H_2SO_4$ . The mixture was kept for three days at  $90^\circ C$  with occasional shaking and the undissolved material was removed by centrifugation. Assay for Ti(IV) by means of  $H_2O_2$  gave 0.58 M. This figure may vary somewhat from one preparation to another which influences the figures 13 min and 6.7 % in the methods described below.

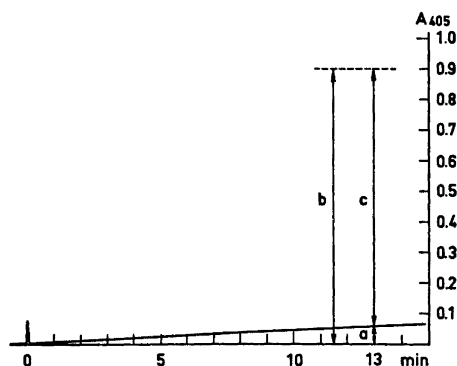
*The standard reagent*. 3.0 ml of water and 50  $\mu l$  Ti stock reagent were mixed in a cuvette. [Ti] will be 9.5 mM and pH 1.18. Only freshly made reagent has been used and no precipitation of titanium hydroxides has been observed.

*The reaction between  $H_2O_2$  and Ti(IV)*.  $H_2O_2$  of known concentration was added to standard reagent.  $A_{405}$  was linear to  $H_2O_2$  in the cuvette (determined for  $A \leq 1.4$ ) giving  $\epsilon \text{ mM} = 0.735 \text{ cm}^2 \text{ mol}^{-1}$  for the complex. The reaction between  $H_2O_2$  and Ti(IV) is instantaneous as recorded by the spectrophotometer.

*Determination of total peroxide*. BHMP, HMP, and  $H_2O_2$ , singly or in combination, was added to a cuvette with 3.0 ml of 50 mM tris-HCl, pH 8.5, to give a concentration of peroxide of 0.5–1 mM. After 5 min incubation at  $25^\circ C$ , 50  $\mu l$  of Ti stock reagent was introduced into the cuvette.  $A_{405}$  was read 6 min after the addition of Ti(IV). Total peroxide was calculated from  $\epsilon \text{ mM} = 0.735 \text{ cm}^2 \text{ mol}^{-1}$ . The absorbance obtained in this manner will henceforth be referred to as the max-absorbance,  $A_{\text{max}}$ .

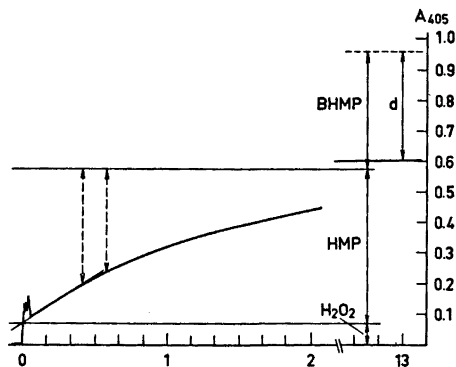
The equilibria in eqns. 2, 3 are far to the left at the conditions in the cuvette. At pH 8.5,  $t_{1/2}$  for the hydrolysis of BHMP is probably less than 15 sec (*vide infra*), and the equilibria (eqns. 2, 3) will soon be reached. Moreover tris reacts with HCHO (*vide infra*) which shifts the equilibria still more to the left with respect to the peroxides. Thus, at the end of the 5 min period, 98–99 % of the peroxide has been converted to  $H_2O_2$ , which reacts instantaneously with the Ti-reagent. The remaining 1–2 % peroxide are present mainly as HMP, which reacts with Ti(IV) nearly quantitatively ( $\geq 98\%$ ) during the adjacent 6 min period (*vide infra*).

*The reaction of BHMP with Ti(IV) standard reagent*. When BHMP is added to standard reagent the solution will slowly turn yellow (Fig. 1a). After 13 min (for choice of time, *vide infra*)  $A_{405}$  (a in the figure) will be 6.7 % of the max. absorbance (b) of the same amount of BHMP or 7.2 % of the difference (c) between a and b.



*Fig. 1a.* The reaction of BHMP in standard reagent. 100  $\mu l$  of 36.6 mM BHMP were added to standard reagent (3 ml  $H_2O$  and 50  $\mu l$  Ti stock reagent). The continuous line shows  $A_{405}$  for this solution, while the dashed line at the top shows the max. absorbance for the same amount of BHMP.

For interpretation: see the text.



*Fig. 1b.* The reaction of a HMP preparation with standard reagent. 100  $\mu l$  "HMP" (total peroxide 41.1 mM) were added to standard reagent (3 ml  $H_2O$  + 50  $\mu l$  Ti stock reagent). The continuous line shows  $A_{405}$  for this solution. The dashed line at the top indicates the max. absorbance for the same amount of "HMP".

The reaction of HMP with Ti(IV) standard reagent. HMP has only been obtained in a mixture with BHMP and  $H_2O_2$ . The changes in  $A_{405}$  upon the addition of an aqueous solution of a HMP preparation to the standard reagent will therefore be composed of several phases (Fig. 1b). The instantaneous increase in absorbance is due to  $H_2O_2$ , while the adjacent slower increase depends mainly upon the reaction of Ti(IV) with HMP and to a lesser degree, BHMP. After 13 min more than 99.9 % of the actually present HMP has reacted (rate constant  $1.05 \times 10^{-2} \pm 0.01$  \*  $\text{sec}^{-1}$ , *vide infra*) but  $A_{405}$  has not reached  $A_{\text{max}}$  for the equivalent amount of hydrogen peroxide. The difference (arrow d, Fig. 1b) depends on the slow reaction of the BHMP present in the preparation (*cf.* Fig. 1a). The absorbance corresponding to [BHMP] in the cuvette ( $A_{\text{BHMP}}$ ) will be 107.2 % of the difference (d) (*cf.* Fig. 1a). Thus the absorbance corresponding to [HMP] in the cuvette will be  $A_{\text{max}}$  minus the instantaneous absorbance (given by  $H_2O_2$ ), minus  $A_{\text{BHMP}}$ . The values for the concentrations of the peroxides obtained by this method are reproducible within less than 2 %.

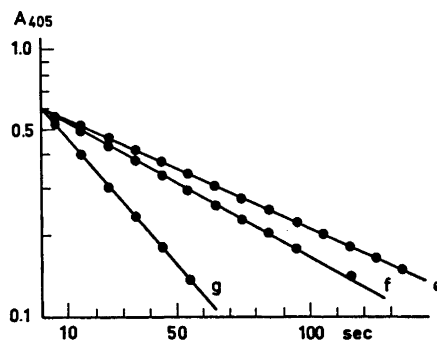
The mechanism of the reaction of HMP with Ti(IV). A semilogarithmic plot of free HMP (not reacted with Ti(IV); see dashed arrows in Fig. 1b) in standard reagent against time gives a straight line (e in Fig. 2). Line f is obtained if the acidity is doubled and g if

Fig. 2. Decrease in free (unreacted with Ti(IV)) HMP at two acidities and Ti concentrations. Initial concentration of HMP = 0.88 mM.

	Ti, mM	pH	Rate constant $\times 10^{-2}$ $\text{sec}^{-1}$
e <sup>a</sup>	9.2	1.18	$1.05 \pm 0.01^b$
f	9.1	0.97	$1.31 \pm 0.01^b$
g	18.1	0.94	$2.72 \pm 0.01^b$

<sup>a</sup> Standard reagent.

<sup>b</sup> S.D., as estimated by the range method.<sup>23</sup>



both the acidity and the Ti(IV) concentration are doubled. The rate constants are given in the legend of the figure. At the pH of the standard reagent, the rate constant of the acid hydrolysis of HMP to  $H_2O_2$  is  $0.008 \times 10^{-2} \pm 10$  % \*  $\text{sec}^{-1}$ , *vide infra*. The yellow colour given by HMP in the standard reagent is consequently mainly due to some direct reaction with Ti(IV) and less so to an acid hydrolysis to  $H_2O_2$ . The influence of the acidity on the reaction (Fig. 2) may be the result of changes in the complexes of Ti(IV).

Pure HMP cannot be synthesized, and it is therefore not possible to measure  $\epsilon$  mM for its compound with Ti(IV) directly. However,  $\epsilon$  mM was assumed to be the same as for titanium- $H_2O_2$  in the methods of determination above, actual results such as the agreement with NMR, the first order decrease of BHMP in the experiments on hydrolysis, and the constancy of the estimated equilibrium constants in Table 3, (*vide infra*) justifying the assumption. It is possible that the compounds of Ti(IV) with  $H_2O_2$ , resp. HMP are identical and that the reaction with HMP goes by way of a hydrolytic fission of  $H_2C(OH)_2$ . It is also possible that the compounds are different in composition but have identical  $\epsilon_{405}$ . The statement that  $\epsilon_{405}$  for HMP-Ti(IV) is larger than that of  $H_2O_2$ -Ti(IV),<sup>24</sup> may be a result of precipitation of titanium hydroxides. The yellow colour given by BHMP in the standard reagent (Fig. 1a), 6.7 % at 13 min of the absorbance of the equivalent amount of  $H_2O_2$ , is fully explained by the reaction of the HMP formed from BHMP by acid hydrolysis (*vide infra*), the value calculated, from the rate of BHMP hydrolysis at pH 1.18 and the rate of the reaction of HMP with Ti(IV), being 6.9 %.

\* S.D., as estimated by the range method.<sup>23</sup>

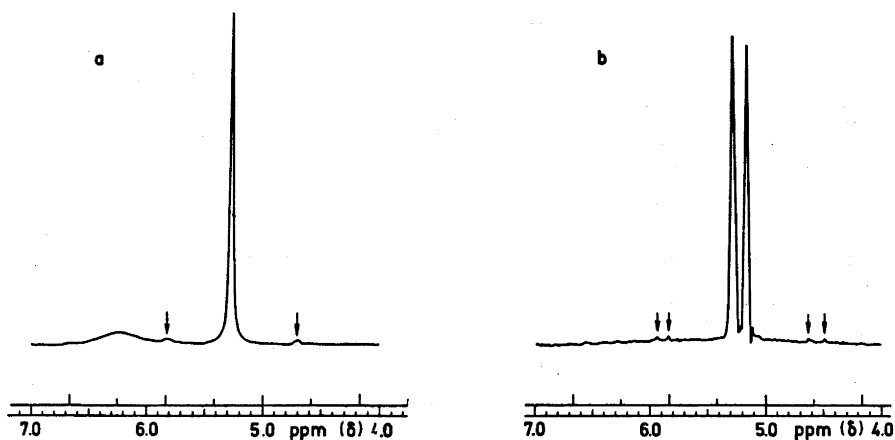


Fig. 3. The NMR spectra of (a) BHMP and (b) a HMP preparation. Arrows indicate spin side bands.

### NMR

At the conditions of synthesis of HMP, a number of organic peroxides in addition to HMP and BHMP could possibly be formed, as mentioned. They would affect the methods of determination described above. NMR spectra were therefore recorded on BHMP and on a HMP preparation to determine whether any other peroxides were present in appreciable extent, and also to check the accuracy of the determination methods.

(a) *BHMP*, Fig. 3a. A sharp peak (probably due to the methylene protons) was found at  $\delta = 5.27$  ppm. The "acid" OH-protons were disseminated between 4.5–7 ppm, with a flat maximum at 6.24 ppm. The ratio between the integrals of the acid protons and the methylene protons ( $1/2.0 \pm 7\%$  \*) was in good agreement with the theoretical one (1:2). A small peak of unidentified origin was found at 3.87 ppm. There was no indication of the crystal water claimed by Serdaroglu.<sup>13</sup>

(b) "*HMP*", Fig. 3b. Two distinct peaks were found, at 5.27 ppm (methylene protons of BHMP), and at 5.15 ppm, probably deriving from the methylene protons of HMP. A batch of the peroxides, prepared with some excess of HCHO, was analyzed by both methods. The ratio HMP:BHMP was according to the integrals of the NMR spectrum 1.44, 1.48, and 1.66 (mean 1.53), whereas the Ti-method gave 1.46 and 1.58 (mean 1.52). The "acid" OH protons were disseminated between 3.5–7.5 ppm. A flat maximum at 11.3 ppm might be ascribed to peroxy protons  $-OOH$  and also possibly  $HOOH$  since it was not found in the spectrum of BHMP. A small peak at 8.5 ppm was due to formic acid ( $HCOOH$ ). There was no sign of formaldehyde in the spectrum. The sum of the methylene proton integrals, the integrals of the  $-OH$ ,  $-OOH$  protons (as calculated from the methylene proton integrals), and the  $-HOOH$  protons (according to Ti determinations) was close to the total integral of the spectrum, the difference being less than 3%.

### EQUILIBRIA BETWEEN THE PEROXIDES

*The base-catalyzed hydrolysis of BHMP* ( $k_{-2}$ , eqns. 3,4). The reaction to the left in eqn. 3 is studied. The rate of disappearance of BHMP (starting with only BHMP and water) was found to depend upon pH of the solution. BHMP was dissolved in 200 mM buffers to give 30 mM solutions, and the changes in composition were determined by the methods described above. The decrease in BHMP followed first order kinetics until about half the amount had vanished, when the back reaction ( $k_2$ ) became influential. Fig. 4a and b

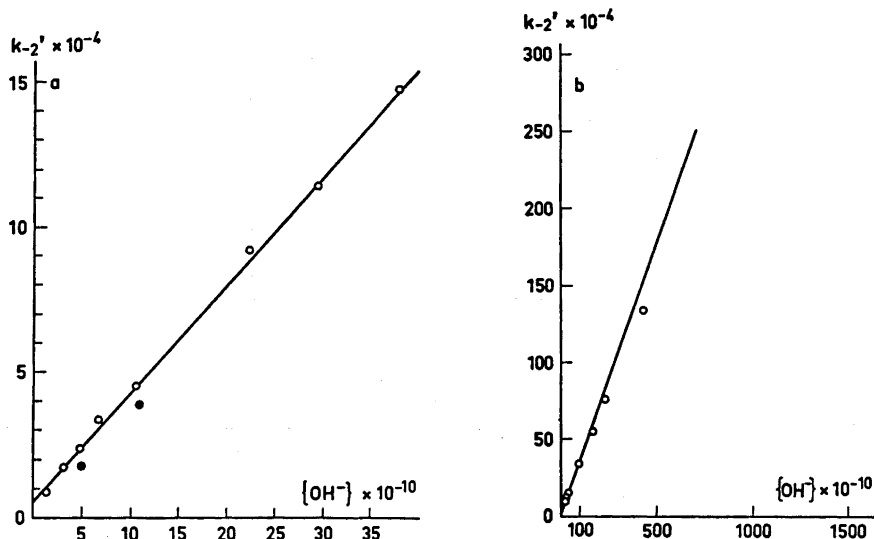


Fig. 4. The dependence of  $k_{-2}'$  on  $\{\text{OH}^-\}$ . The calculations of  $k_{-2}'$  and  $\{\text{OH}^-\}$  are described in the text. The circles represent from the left in a: 200 mM sodium acetate pH 4.12, 4.49, 4.67, 4.82, 5.02, 5.34, 5.47, and 5.58. The solid circles 50 mM sodium acetate pH 4.69, 5.03. b: 200 mM sodium acetate pH 5.34, 5.47, 5.58, 200 mM sodium cacodylate pH 5.98, 6.22, 6.36 and 6.63. The continuous lines are the same in both figures.

give the variation in  $k_{-2}'$  by  $\{\text{OH}^-\}$ , the latter calculated from pH and  $\text{p}K_w = 13.996$  at  $25^\circ\text{C}$ .<sup>20</sup>

*The base catalyzed hydrolysis of HMP ( $k_{-1}'$ , eqns. 2, 4).* The effect of  $\{\text{OH}^-\}$  on HMP preparations was studied by following the changes in composition in different buffers as above. The calculation of  $k_{-1}'$  however, was complicated by the contribution of HMP from BHMP.  $k_{-1}'$  could not be conveniently determined from the over-all rate of formation of  $\text{H}_2\text{O}_2$  and the known value for  $k_{-2}'$  because of the early appearance of back reactions. Instead, the procedure in Fig. 5 was adhered to. The hydrolysis of HMP was followed in terms of  $\text{H}_2\text{O}_2$  increases in solution. The batch of HMP used in the experiment contained  $\text{H}_2\text{O}_2$  to give an initial absorbance around 0.06. The increase in  $\text{H}_2\text{O}_2$  up to  $A \approx 0.12$  was approximately linear. The points in the figure could therefore be connected with a straight line. Within the same time [HMP] remained approximately constant because of the contribution from BHMP. The rate constant of the hydrolysis of HMP ( $k_{-1}'$ ) was taken as  $k_{-1}' = i/t \bar{h}$  ( $t$  in sec,  $\bar{h}$  the mean value of  $h_1 \cdots h_n$ )  $k_{-1}'$  as obtained in this way is somewhat too small because of the influence of the back reaction ( $\text{H}_2\text{O}_2 + \text{HCHO} \rightarrow \text{HMP}$ ). This effect has been approximated to  $< 10\%$ . The values of  $k_{-1}'$  are not corrected for this deviation. The dependance of  $k_{-1}'$  on  $\{\text{OH}^-\}$  is given in Fig. 6.

*The acid-catalyzed hydrolysis of BHMP ( $k_{-2}'$ ) and HMP ( $k_{-1}'$ ).* The experiments were performed in the same manner as the experiments on the  $\text{OH}^-$ -catalysis. Figs. 7 and 8 show the rate constants ( $k_{-2}'$ ,  $k_{-1}'$ ) plotted against hydrogen ion activities.

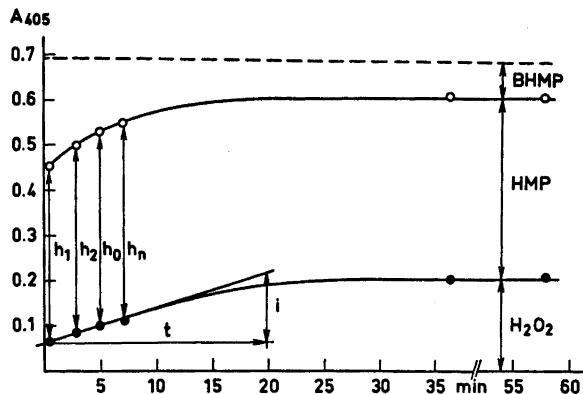


Fig. 5. The determination of  $k_{-1}'$ . The figure illustrates the reaction at pH 5.62. 10  $\mu$ l of a HMP preparation were dissolved in 5 ml of 200 mM sodium acetate (pH 5.62), 25°C. Samples (100  $\mu$ l) were transferred to standard reagent and tris buffer to follow the changes in composition. The results are represented by the points in the figure. The symbols are explained in the text. The dashed line at the top, representing max. absorbance, is declining slightly because of a side reaction, probably the formation of HCOOH.

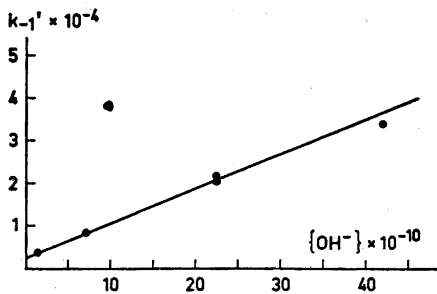


Fig. 6. The base catalyzed hydrolysis of HMP. The method for the calculations of  $k_{-1}'$  and  $\{\text{OH}^-\}$ , is described in the text. The points represent from the left: 200 mM sodium acetate pH 4.13, 4.85, 5.35 (two points) and 5.62.

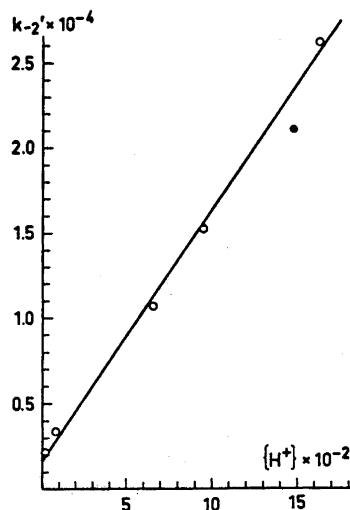


Fig. 7. The acid catalyzed hydrolysis of BHMP.  $k_{-2}'$  was calculated as described in the text. The circles represent from the left: 200 mM sodium sulphate pH 2.77, 2.11, 1.18, 1.03, and 0.79. The solid circle 200 mM HClO<sub>4</sub> pH 0.83.

*The stabilities of BHMP and HMP.* Fig. 9 presents the stabilities of BHMP and HMP in a comprehensible way.  $k_{-2}'$  above pH 5.6 can be obtained from Fig. 4b.



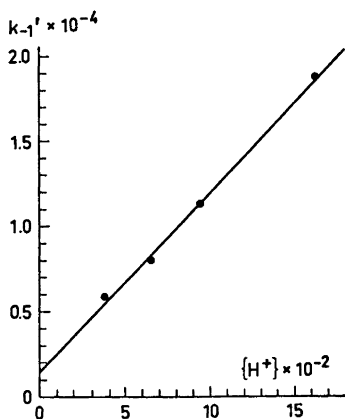


Fig. 8. The acid catalyzed hydrolysis of HMP. 10  $\mu$ l of a HMP preparation were dissolved in 5 ml of buffer, and 100  $\mu$ l samples were brought to standard reagent and tris after measured time intervals. The rate constants of the hydrolysis were calculated as described in the text. The points represent from the left: 200 mM sodium sulphate, pH 1.42, 1.18, 1.03, and 0.79.

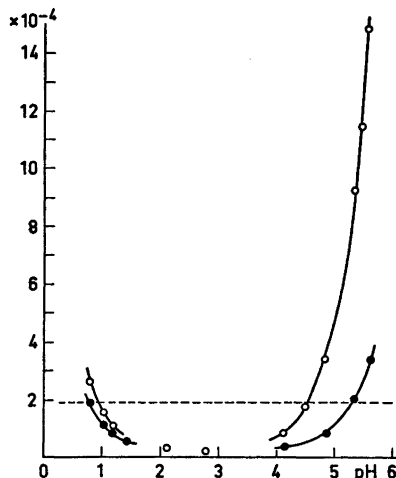


Fig. 9. pH dependence of the rate constants for the hydrolysis of (O) BHMP ( $k_{-2}'$ ) and (●) HMP ( $k_{-1}'$ ).

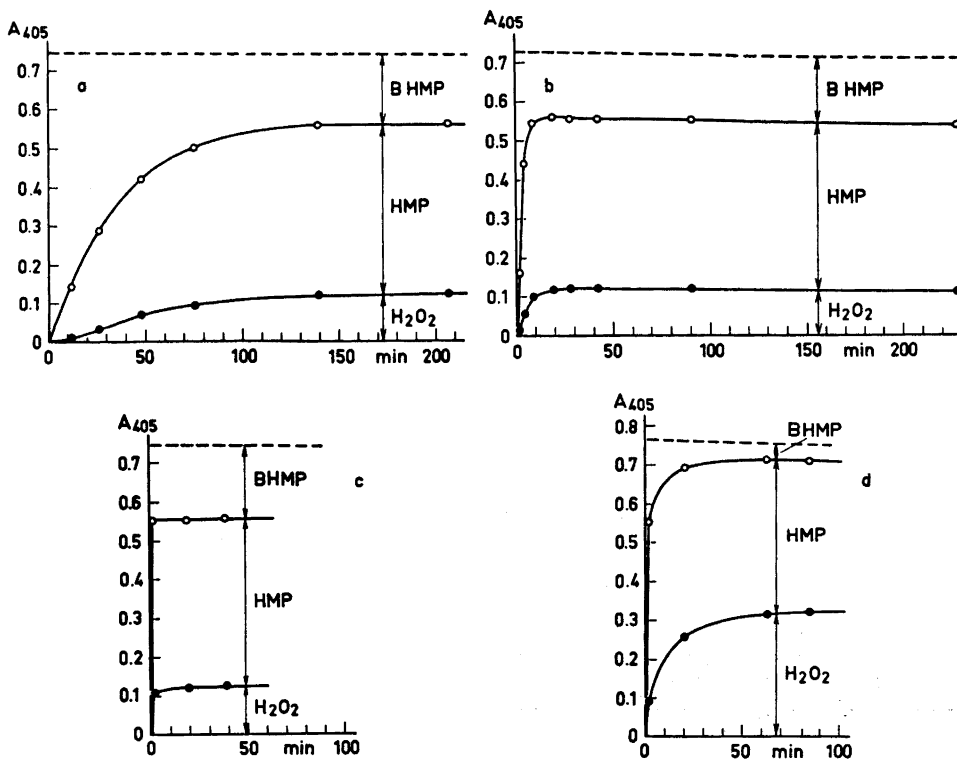
**Equilibria.** In solutions of  $\text{H}_2\text{O}_2 + \text{HCHO}$  or peroxides derived from them, equilibria will be attained. The rate at which the equilibria are reached, depends on pH, *i.e.* the  $\text{OH}^-$  and  $\text{H}^+$  activities.

Fig. 10a–d shows the events in solutions of BHMP at different pH's.

The equilibrium constants are given in Table 2. The above equilibrium is not obtained in the presence of tris (Fig. 10d). The deviation suggests that tris reacts with HCHO. This reaction is utilized in the determination of total peroxide (above).

Table 2. The equilibrium constants at different pH's.  $K_1'$  and  $K_2'$  are defined in eqns. 2, 3 and 4. They were calculated from the concentrations of the peroxides and HCHO at equilibrium. The latter was obtained as the difference between HCHO initially bound as BHMP and HCHO bound as BHMP + HMP at equilibrium.

pH	$K_1'$ $\text{M}^{-1}$	$K_2'$ $\text{M}^{-1}$
4.75	125	13.3
6.0	132	14.4
7.19	121	14.2
$\bar{m}$	$126 \pm 6$ (S.D.)	$14.0 \pm 0.6$ (S.D.)



*Fig. 10.* The reach of equilibria in different buffers with BHMP as starting point. BHMP was dissolved in different buffers to give concentrations of around 32 mM. Samples of 100  $\mu$ l were transferred to standard reagent and tris at suitable intervals to determine the changes in composition. The results are presented as absorbances at 405 nm. The dashed lines at the top, representing max. absorbances, decline slightly because of a side reaction, probably the formation of HCOOH. a. 200 mM sodium acetate (pH 4.75). b. 200 mM sodium cacodylate (pH 6.0). c. 200 mM sodium cacodylate (pH 7.19). d. 200 mM tris-HCl (pH 7.19). The equilibrium constants in a, b, and c are presented in Table 2.

*Table 3.* The equilibrium constants  $K_1'$  and  $K_2'$  at different concentrations of total peroxide. BHMP was dissolved in 200 mM Na acetate (pH 5.5, 25°C) to achieve the in the table presented concentrations. When equilibrium was obtained (after about 2 h samples (0.1 ml) were transferred to standard reagent and tris buffer to determine the composition of peroxides. HCHO was obtained as the difference between HCHO initially bound as BHMP and HCHO bound as BHMP + HMP at equilibrium.

Initial BHMP mM	$K_1'$ M <sup>-1</sup>	$K_2'$ M <sup>-1</sup>
15.7	124; 128	12.9; 16.1
27.6	121; 125	13.7; 14.3
64.5	126; 129	14.9; 14.6
$\bar{m}$	126 $\pm$ 3 (S.D.)	14.4 $\pm$ 1.1 (S.D.)

The equilibria have also been investigated at constant pH with different concentrations of BHMP (Table 3). Equilibria of the same order of magnitude are obtained at pH around 1, too, but were not systematically studied.

Kooijman and Ghijsen,<sup>16</sup> who determined the equilibrium constants in acid solution by means of freezing point depressions, found  $K_1' = 94.3 \text{ M}^{-1}$  and  $K_2' = 14.7 \text{ M}^{-1}$ .

*The rate constants of formation of HMP and BHMP.* The equilibrium constants  $K_1'$  and  $K_2'$  (eqns. 2, 3, 4) were found to be essentially unchanged over a wide range of pH (4.75–7.19 (Table 2)). As the rates of hydrolysis ( $k_{-1}'$ ,  $k_{-2}'$ ) are known, the constants of formation are obtained as  $k_1 = K_1'k_{-1}'$ , and  $k_2 = K_2'k_{-2}'$ .  $k_1$  was also experimentally determined at one pH. A solution of HCHO, properly depolymerized, and  $\text{H}_2\text{O}_2$  in 67 mM sodium acetate (pH

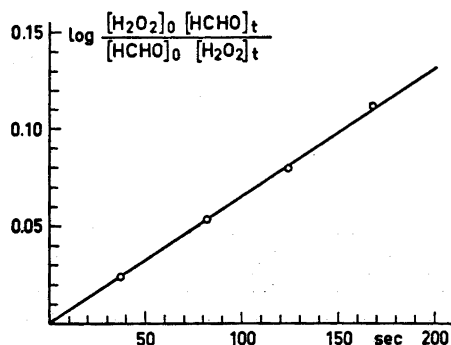


Fig. 11. Determination of the rate constant of the formation of HMP ( $k_1$ ). Formaldehyde and hydrogen peroxide were mixed in 67 mM sodium acetate, pH 5.49, to give  $[\text{HCHO}] = 63.0 \text{ mM}$  and  $[\text{H}_2\text{O}_2] = 38.6 \text{ mM}$ , samples ( $100 \mu\text{l}$ ) being assayed in standard reagent or tris at given times. The decrease in  $[\text{HCHO}]$  was assumed to parallel the decrease in  $[\text{H}_2\text{O}_2]$ . As only the initial part of the reaction was followed, the formation of BHMP was small and negligible.

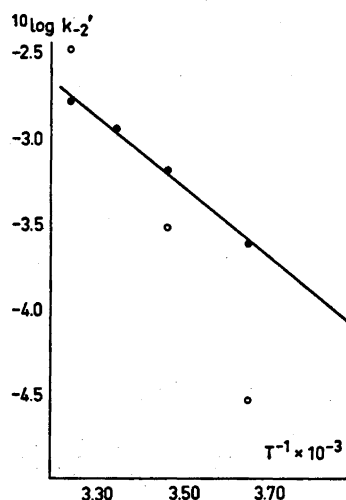


Fig. 12.  $\log k_{-2}'$  plotted against  $1/T$ . Determination of the activation energy of the  $\text{OH}^-$ -catalyzed hydrolysis of BHMP. For interpretation see the text.

5.49) was prepared and the changes in composition determined with the Ti(IV) method. A plot of  $\log (\text{H}_2\text{O}_2/\text{HCHO})_0 \times (\text{HCHO}/\text{H}_2\text{O}_2)_t$  against time (Fig. 11) resulted in a straight line — establishing second order kinetics — with a slope corresponding to  $k_1 = 31.2 \times 10^{-3} \text{ mol}^{-1} \text{ sec}^{-1} \pm 2 \%$ . \* The value of  $k_1$ , obtained from  $K_1'$  ( $126 \text{ M}^{-1}$ ) and  $k_{-1}'$  ( $2.5 \times 10^{-4} \text{ sec}^{-1}$ , recalculated from pH 5.62) is

$31.5 \times 10^{-3} \text{ mol}^{-1} \text{ sec}^{-1} \pm 11 \%$ . \* The agreement between the two  $k_1'$ -values exclude side reactions. This determination was made at a pH where basic catalysis is predominant (cf. Fig. 9). The reaction has previously also been investigated in acid solution<sup>25</sup> and reported to be 2/3 order with respect to HCHO and first with respect to  $\text{H}_2\text{O}_2$ . This may be a result of inadequate determination methods and influence of the formation of BHMP ( $k_2$ ) and the back reaction ( $k_{-1}$ ).

*The energy of activation of the  $\text{OH}^-$ -catalyzed hydrolysis of BHMP.* Of interest from a biochemical point of view is the temperature effect on the  $\text{OH}^-$ -catalyzed hydrolysis. BHMP was added to 200 mM sodium acetate ( $0^\circ$ , pH 5.49), ( $14.8^\circ$ , pH 5.48), ( $25^\circ$ , pH 5.47), and ( $35^\circ$ , pH 5.46), and the rate constants ( $k_{-2}'$ ) of BHMP hydrolysis were determined as described above.  $\{\text{OH}^-\}$  was calculated from  $K_w$  at the temperature and the actual pH,<sup>20</sup> but no compensations were made for the ionic strength of the buffer. For the calculation of the energy of activation all rate constants were transformed to the same  $\{\text{OH}^-\}$ , by assuming a linear relationship between  $k_{-2}'$  and  $\{\text{OH}^-\}$ . Fig. 12 shows  $\log k_{-2}'$  plotted against  $1/T$ . Empty circles represent untransformed values of  $k_{-2}'$ . The straight line in the figure corresponds to an energy of activation of 39 kJ mol<sup>-1</sup>. The slight deviations of the points from the straight line may partly be caused by different influences of the ionic strength of  $K_w$  at different temperatures. The above assumption of a linear relationship between  $k_{-2}'$  and  $\{\text{OH}^-\}$  is not quite correct and may also have influenced.

## DISCUSSION

One purpose of this work was to find a method to quantify BHMP, HMP, and  $\text{H}_2\text{O}_2$  in water mixtures. NMR could only be used with deuteriated solvent and at rather high peroxide concentrations. A previously reported, chromatographic method<sup>26</sup> was tried but found unsuitable for quantitative analysis; even qualitative use was difficult because of the hydrolysis of HMP and BHMP and the volatility of  $\text{H}_2\text{O}_2$ . Polarograms of the peroxides have been presented,<sup>27</sup> but they appear to give less precision when used for determinations. The "titanium method" described in this paper is reasonably fast (less than 30 min), has a good precision ( $\pm 2 \%$ ) and accuracy as seen in the comparison with NMR. It may be adaptable for the analysis of peroxide systems similar to  $\text{H}_2\text{O}_2$ , HMP, and BHMP.

The second purpose was to find out whether organic peroxides in addition to HMP and BHMP would be formed during the synthesis of HMP, or else when the peroxides are kept in water solution. In the NMR spectrum of "HMP" (Fig. 3b) no such peroxides could be detected. Moreover the interpretation of the experimental results on the basis of kinetic and equilibrium data does not require the introduction of any compounds in addition to HCHO,  $\text{H}_2\text{O}_2$ , HMP, and BHMP.

The third purpose was to find out the stabilities of BHMP and HMP in order to get controlled conditions in enzyme studies. The experiments show that BHMP and HMP are rapidly hydrolyzed at conditions frequently used

in enzyme studies (see Fig. 9),  $t_{1/2}$  for BHMP at pH 7 being around 30 sec at room temperature. It is obvious therefore that the effects ascribed to BHMP in earlier studies could well have been produced by HMP.

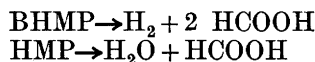
The experiments show a linear relationship between the rates of hydrolysis and the activities of  $\text{OH}^-$  and  $\text{H}^+$  (Figs. 4a, b, 6, 7, 8). The pseudo-first-order rate constants of hydrolysis are supposed to be composed as:

$$\begin{aligned}
 k &= k_0 + k_{\text{H}}\{\text{H}^+\} + k_{\text{OH}}\{\text{OH}^-\} + k_{\text{HA}}\{\text{HA}\} + k_{\text{A}}\{\text{A}^-\} \\
 k_0 &= \text{the rate constant of the spontaneous reaction} \\
 k_{\text{H}} &= \text{the catalytic constant for } \text{H}^+ \\
 k_{\text{OH}} &= \text{the catalytic constant for } \text{OH}^- \\
 k_{\text{HA}} &= \text{the catalytic constant for the acid HA} \\
 k_{\text{A}} &= \text{the catalytic constant for the base } \text{A}^-
 \end{aligned}$$

The catalytic constants for  $\text{H}^+$  and  $\text{OH}^-$  can be determined from the slope of the lines in Figs. 4a, b, 6, 7, 8. The  $k_{\text{H}}$  and  $k_{\text{OH}}$  are indeed small enough to make the  $\text{H}^+$ -catalysis negligible under conditions which render the  $\text{OH}^-$ -catalysis appreciable and *vice versa*. As seen from the effect of a reduction of the buffer concentration from 200 mM to 50 mM (Fig. 4a), there is some activity of buffer acid and base, the latter appearing to be predominant. (It is not possible from these experiments to conclude whether the sodium ions or the acetate base cause the hydrolysis, but the high sensitivity of the peroxides towards another base ( $\text{OH}^-$ ) suggests that the acetate base is the active species.) In the pH-range below 5.5 this buffer effect may be of significance; acid and basic groups in proteins in solution could have a similar effect. The straight lines in the figures (Figs. 4a, b, 6, 7, 8) do not pass through the origin. This seems to be due to the hydrolysis caused by the buffers; the spontaneous reaction ( $k_0$ ) is probably negligible. Since the constants of equilibria are essentially constant over a wide range of pH (Table 2), it is obvious that the formation of BHMP and HMP is catalyzed by base, and presumably also by acid.

Tris appears to change the equilibria (eqns. 2, 3, Fig. 10d). The effect can be attributed to a reaction between the amino group of tris and formaldehyde. Therefore when working with the peroxides at equilibrium in various media, attention should be given to a possible influence of amino groups in proteins.

In addition to the above hydrolyses other reactions may occur, which yield formate. Their mechanisms have been given as:<sup>10,15</sup>



These processes proceed rapidly at high alkalinity (pH > 12) but very slowly in neutral solutions. The decrease in total peroxide seen in Figs. 5 and 10 is probably caused by such reactions, and so is the presence of HCOOH in HMP preparations as seen in NMR. When BHMP or a HMP preparation is dissolved in water, a pH around 3.5–4 is obtained. The acid is probably HCOOH, derived from the above reactions. At this acidity the peroxides are rather stable (*cf.* Fig. 9) and at 0°C the rates of hydrolysis will be very low as seen

from the activation energy. Solutions of the peroxides can thus be kept on ice for hours without much change in composition.

Rieche and Meister<sup>15</sup> claimed the preparation of "pure" HMP under anhydrous conditions in ether. In the method of synthesis in ether described in the present paper, the formaldehyde is in the form of  $\text{H}_2\text{C}(\text{OH})_2$ . The "anhydrous" method has not been tried here, but it seems unreasonable to suppose that it would produce HMP without the simultaneous formation of BHMP and the equilibria (eqns. 2, 3). The analytical methods used by Rieche and Meister to show the purity of their HMP, cannot distinguish the latter from the 1:1 mixture of peroxides in Table 1. These authors calculated the molecular weight from experiments on the freezing point depression. The mole fractions in the 1:1 preparation (Table 1) are 0.14 for  $\text{H}_2\text{O}_2$ , 0.14 for BHMP, and 0.72 for HMP, since there is no free HCHO; thus the equivalent weight is the same as that of pure HMP. They also determined the contents of "active oxygen", and again "pure HMP" and the "1:1 mixture" would produce equal results.

*Acknowledgements.* The project was proposed by Prof. K.-G. Paul, and I wish to thank him for his advice, criticism, and discussion. I also wish to express my gratitude to Mr. B. Långström for help with NMR, to Dr. S. Wold for discussion, and to Mrs. Siw Öhman for technical assistance. The study was supported by *Statens naturvetenskapliga forskningsråd* (320-7, 7926 K).

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Received March 12, 1971.