Phosphorylation Coupled to Electron Transfer in Aqueous Solutions*

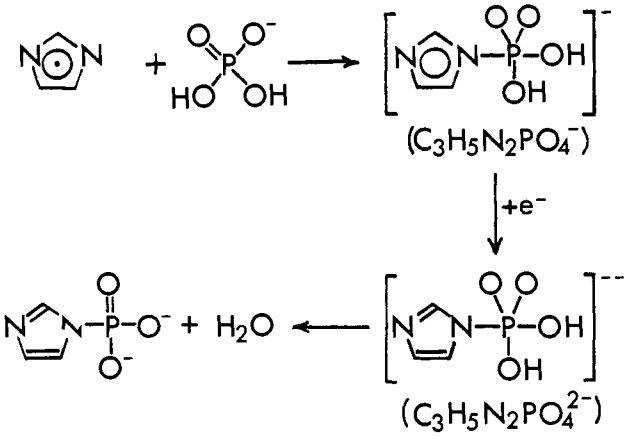
Shu-I Tu and Jui H. Wang

ABSTRACT: Visible light absorbed by hematoporphyrin is utilized to drive the condensation of imidazole with inorganic orthophosphate in aqueous solution to form 1-phosphoimidazole.

The molecular mechanism for coupling this light-driven electron transfer to the observed phosphorylation reaction is further examined by trapping the radical intermediates and measuring their electron spin resonance spectra.

In our previous studies of model oxidative phosphorylation systems, it was found that when ferrohemochrome was oxidized by air in *N,N*-dimethylacetamide solution containing AMP, P_i, and imidazole, ADP and ATP are formed (Brinigar *et al.*, 1967). Subsequent work with substituted imidazoles (Cooper *et al.*, 1968) suggests that O_2 first extracts two electrons from the ferrohemochrome to produce a complex of Fe^{III}-heme and the reactive imidazolyl radical ($C_3H_3N_2$). The imidazolyl radical can then react rapidly with P_i to form an unstable phosphoimidazolyl radical, which is subsequently reduced by another ferrohemochrome molecule to produce 1-phosphoimidazole and water (see Scheme I). The formation

SCHEME I



of the trigonal–bipyramidal intermediate compound 1-orthophosphoimidazole ($C_3H_5N_2PO_4^{2-}$) by the usual nucleophilic attack at the P atom is not only very slow but thermodynamically unfavorable. But since radical reactions generally require a much lower activation free energy, the trigonal–bipyramidal phosphoimidazolyl radical ($C_3H_5N_2PO_4^-$) can be formed much more rapidly (through radical addition to the P=O double bond). In the subsequent step driven by the oxidation–reduction free energy, this phosphoimidazolyl radical is reduced to the unstable 1-orthophosphoimidazole which then spontaneously eliminates H_2O to form 1-phosphoimidazole. In this way, electron transfer can be coupled to phosphorylation.

In the present work, we report that the same molecular mechanism can be utilized to couple light-driven electron transfer to phosphorylation in aqueous solutions in the absence of molecular oxygen. Some of the radical intermediates can be trapped in frozen samples and characterized by their electron spin resonance spectra.

Experimental Section

Materials. Imidazole (Grade III), adenosine-5′-phosphoric acid (Sigma Grade, Type IV), hematoporphyrin dihydrochloride, and firefly lantern extract were from Sigma Chemical Co. 2,4-Dimethylimidazole was from Gallard-Schlesinger Corp. and was recrystallized from hot ethanol (mp 88°). Pyrazole from Aldrich Chemical Co. was recrystallized from hot benzene (mp 69–70°).

1-Phosphoimidazole was synthesized according to the procedure of Rathlev and Rosenberg (1956). The deuterium oxide (99.7%) and imidazole-d_4, supplied by Merck, Sharp and Dohme Co., did not show any detectable proton resonance signal under the conditions of our nuclear magnetic resonance measurements. Spectro Analyzed *N,N*-dimethylacetamide (Matheson, Coleman & Bell) was further purified by passing through a composite alumina column as reported before (Cooper *et al.*, 1968). All the other chemicals used were of analytical reagent quality. Deionized water with 19×10^6 ohms resistivity was used to make all aqueous solutions.

Illumination. In the auxiliary experiments, an aqueous solution of hematoporphyrin (1.4×10^{-5} M) and imidazole (1.5×10^{-3} M) in 0.1 M phosphate buffer (pH 7.0) was sealed under vacuum in glass tubes and illuminated at 10° with 150-W tungsten lamp. A yellow filter which transmitted less than 0.1% of the incident light of wavelengths below 450 nm was used. In the more quantitative experiments, the vacuum-sealed sample was illuminated at 2 ± 1° with a 500-W projector lamp. No detectable alteration in the absorption spectrum of the hematoporphyrin was observed after the illumination period.

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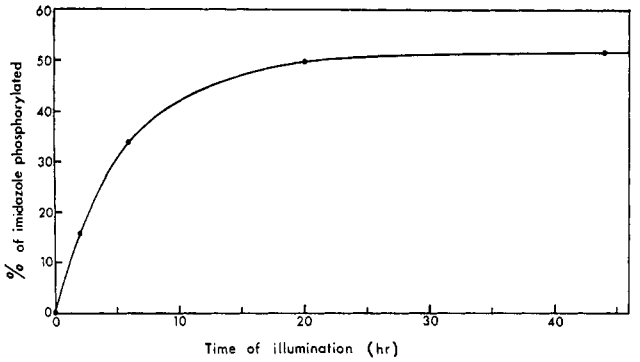


FIGURE 1: Phosphorylation of imidazole coupled to the light-driven electron transfer in aqueous phosphate buffer (pH 7.0).

ATP Assay. For the auxiliary experiments, the illuminated aqueous mixtures were pumped to dryness under vacuum at room temperature. The residue, which contains the phosphorylated imidazoles, was incubated with a *N,N*-dimethylacetamide solution of AMP at room temperature in the dark. The concentration of ATP formed was determined from time to time by the firefly method (Brinigar *et al.*, 1967).

Separation and Determination of 1-Phosphoimidazole. For the more quantitative experiments, the phosphorylated imidazoles in the illuminated aqueous solution were assayed directly without further reaction with AMP. Imidazole and 1-phosphoimidazole were determined by the methods of Macpherson (1942) and of Rathlev and Rosenberg (1956). In a typical assay, the sealed aqueous sample was opened to air after the light was turned off. The pH of the solution was adjusted to 12 with 1.0 M NaOH solution. To a 2-ml aliquot of this sample, 5 ml of 0.2 M $CaCl_2 \cdot 2H_2O$ in ethanol solution was added. The mixture was stirred for 20 min to pecipitate the hematoporphyrin, inorganic orthophosphate, inorganic pyrophosphate, and phosphorylated imidazoles. After removing the precipitate by centrifugation, the concentration of imidazole was determined colorimetrically by diazotized sulfanilic acid. Calibration experiments with known mixtures of imidazole and synthetic 1-phosphoimidazole showed that imidazole was not precipitated under those conditions. The colorimetric calibration curve obtained with a Gilford Automatic recording photometer is linear with an experimental uncertainty of $\pm$ 1% for imidazole concentration in the range 0–1 $\times 10^{-3}$ M. The washed precipitate was treated with dilute HCl to hydrolyze the phosphoimidazole to imidazole and P_i. Then after precipitating the inorganic phosphate and hematoporphyrin with $CaCl_2 \cdot 2H_2O$ solution and centrifuging as before, the concentration of imidazole in the supernatent was again determined colorimetrically. This last value represents the molar concentration of phosphorylated imidazole, and is found equal to the observed decrease in the concentration of free imidazole within an experimental error of $\pm$4%.

Electron Spin Resonance Measurements. A Varian E-3 EPR spectrometer was used for the magnetic resonance measurements. For the measurements in Figure 2, the cavity temperature was preadjusted to $-150°$ before the frozen sample was put in. For the measurements in Figure 3, the samples were immersed in liquid nitrogen during the measurements.

Results

Auxiliary Experiments. Separation by paper chromatography as in the previous work (Brinigar *et al.*, 1967) showed that the illuminated aqueous mixture contains the phosphorylated imidazole in addition to inorganic orthophosphate and the imidazole. If imidazole was used, the phosphorylated imidazole has the same R_F value as 1-phosphoimidazole. Incubation of this intermediate with AMP in *N,N*-dimethylacetamide solution gave ATP. In a typical experiment, an aqueous solution of hematoporphyrin (1.4×10^{-5} M) and imidazole (1.5×10^{-3} M) in phosphate buffer (0.1 M) at pH 7.0 was illuminated in the absence of air with yellow light at 10° for 2 days. The water was then removed and replaced with an equal volume of *N,N*-dimethylacetamide solution of AMP (1.1×10^{-3} M). After the mixture was incubated for 7 days at room temperature in the dark, it was found to contain 3.5×10^{-5} M of ATP. An unilluminated control sample gave no detectable amount of ATP after incubation.

When the imidazole in the above experiment was replaced by either 2,4-dimethylimidazole or pyrazole, ATP was also formed. Presumably the intermediate formed in these cases were 1-phospho-2,4-dimethylimidazole and 1-phosphopyrazole, respectively. But, as expected from our previous work, no ATP was formed when 1-methylimidazole was used to replace imidazole in these experiments.

Quantitative Study. The observed production of 1-phosphoimidazole in an illuminated aqueous solution containing hematoporphyrin (2.0×10^{-4} M) and imidazole (1.0×10^{-3} M) in inorganic orthophosphate buffer (0.1 M) at pH 7.0 and 2° is summarized in Figure 1. Under the experimental conditions, the concentration of 1-phosphoimidazole apparently reached half of its maximum concentration within 4-hr illumination.

Radical Intermediates. When a solution of hematoporphyrin (HP) and a suitable electron donor in aqueous phosphate buffer (pH 7) was frozen at $-150°$ and illuminated with yellow light in the absence of imidazole, the sample did not show any detectable electron spin resonance signal during the first 30 sec, but then an electron spin resonance signal at g = 2.0026 (half-width, 7.5 G) gradually appeared and grew to substantial intensity over a period of about 20 min. This electron spin resonance signal was identical with that of the reduced hematoporphyrin radical characterized previously (Eisenstein and Wang, 1969).

By contrast, when a solution of hematoporphyrin and imidazole in phosphate buffer at pH 7 was frozen at $-150°$ and illuminated by yellow light, and electron spin resonance signal g = 2.0036 (half-width, 6.1 G) was detected immediately (within 1 sec). We attribute this fast electron spin resonance signal to the imidazolyl radical ($C_3H_3N_2$), since its line width decreased to 5.5 G when the normal imidazole was replaced by deuterated imidazole (imidazole-d_3 or -d_4). These experimental results are summarized in Table I. The electron spin resonance spectra of frozen samples containing imidazole or its analogues are summarized in Figure 2. As expected from our previous work (Cooper *et al.*, 1968), the illumination of frozen samples of hematoporphyrin + imidazole, hematoporphyrin + 2,4-dimethylimidazole, and hematoporphyrin + pyrazole, respectively, yielded the imidazolyl radical-type of electron spin resonance spectra, and the illumination of a frozen sample of hematoporphyrin + 1-methylimidazole did

TABLE I: Light-Induced Electron Spin Resonance Signals in Aqueous Mixtures at −150°.

Generating Reaction[a]	G Value	Line Width (G)
$HP + HPh \xrightarrow[slow]{h\nu}$	2.0026	7.5
$HP + EDTA \xrightarrow[slow]{h\nu}$	2.0026	7.5
$HP + imidazole \xrightarrow[fast]{h\nu}$	2.0036	6.1
$HP + imidazole\text{-}d_4 \xrightarrow[fast]{h\nu}$	2.0036	5.5[b]

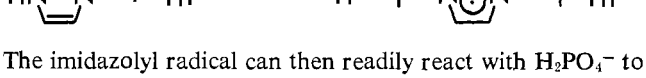
[a] Abbreviations used are: HP, hematoporphyrin; HPh, hematophlorine. [b] Same value in either H_2O or D_2O solution.

not. The imidazole radical trapped in frozen samples is unstable in liquid solution, since its electron spin resonance spectrum disappeared completely when the sample was melted for even a few seconds and then quickly refrozen.

After the frozen hematoporphyrin + imidazole in phosphate buffer sample was illuminated for many hours, side bands began to appear in the electron spin resonance spectrum. The generation of these side bands can be accelerated by preilluminating the liquid sample with yellow light above 0°. If the preilluminated sample was frozen in the dark, no electron spin resonance spectrum was detectable. But subsequent illumination in the frozen state at −150° readily generated the complex electron spin resonance spectrum shown in Figure 3A. The strong single derivative peak in Figure 3A is due to imidazolyl radical in the mixture, since it is identical with the electron spin resonance spectrum shown in Figure 2A. The complex spectrum in Figure 3A is probably due to a radical species produced through the reaction of 1-phosphoimidazole, since its intensity increases with the concentration of 1-phosphoimidazole produced during the preillumination period. This conclusion is further supported by the observation that by freezing a solution of hematoporphyrin and synthetic 1-phosphoimidazole and illuminating with yellow light, the electron spin resonance spectrum in Figure 3B, which is identical with that of Figure 3A, appeared within a few seconds. Figure 3C shows the electron spin resonance spectrum of a sample prepared by a procedure similar to that for Figure 3A but with pyrazole replacing the imidazole.

Discussion

The above experimental results are entirely consistent with the radical mechanism for coupling phosphorylation to electron transfer. With the electronically excited hematoporphyrin as the oxidant, the initial step becomes



The imidazolyl radical can then readily react with $H_2PO_4^-$ to form phosphoimidazolyl radical ($C_3H_5PO_4^-$) which can sub-

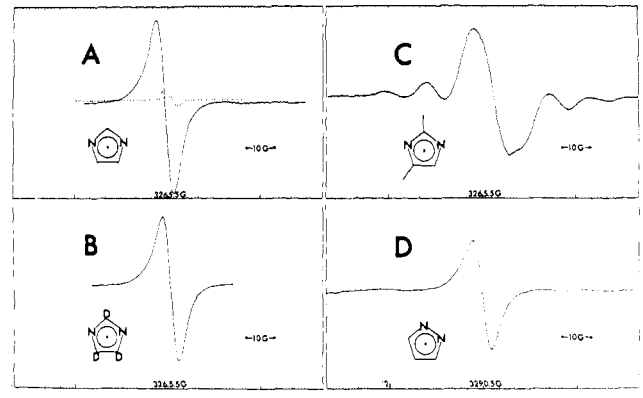


FIGURE 2: Electron spin resonance spectra of photogenerated imidazolyl and related radicals trapped in frozen mixtures at −150°. All samples contain 2.0 × 10^{-4} M hematoporphyrin in 0.1 M phosphate buffer (pH 7.0). Additional reactant in each sample: (A) imidazole (1.5 × 10^{-2} M) (solid curve, frozen sample under illumination; broken curve, sample melted for a few seconds and refrozen in the dark, residual signal due to the empty electron spin resonance tube); (B) imidazole-d_3 or -d_4 (1.5 × 10^{-2} M); (C) 2,4-dimethylimidazole (1.5 × 10^{-2} M; (D) pyrazole (1.5 × 10^{-2} M). The microwave frequencies are (A) 9.148, (B) 9.148, (C) 9.160, and (D) 9.223 GHz, respectively.

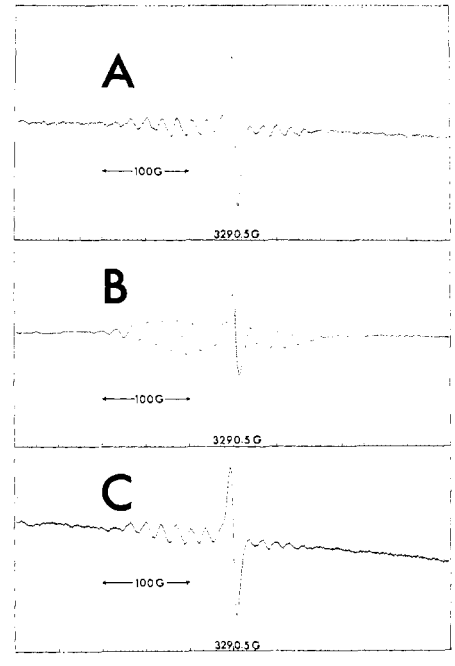


FIGURE 3: Electron spin resonance spectra of radicals derived from 1-phosphoimidazole or 1-phosphopyrazole in frozen mixtures at −196°. All samples contain 2.0 × 10^{-4} M hematoporphyrin in 0.1 M phosphate buffer (pH 7.0). Special features of each experiment: (A) The mixture containing 1.5 × 10^{-2} M imidazole was illuminated by yellow light in an evacuated and sealed tube for 48 hr at room temperature, subsequently frozen in liquid nitrogen and again illuminated for a few minutes, then the light was turned off, and the electron spin resonance spectrum was taken in the dark at −196°. Microwave frequency, 9.219 GHz. (B) The mixture containing 9.0 × 10^{-3} M 1-phosphoimidazole was sealed in an evaluated tube, frozen in liquid nitrogen, and illuminated by yellow light for a few minutes, then the electron spin resonance spectrum was taken in the dark at −196°. Microwave frequency, 9.222 GHz. (C) Same as part A except the pyrazole was used instead of imidazole. Microwave frequency, 9.221 GHz.

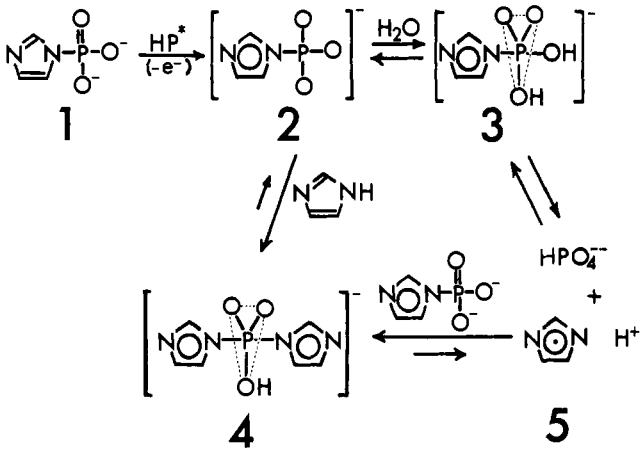


FIGURE 4: A mechanistic scheme for the probable reactions which generate the phosphoimidazolyl radicals in the model system for coupling phosphorylation to light-driven electron transfer.

sequently be reduced by the HP^- radical to form 1-phosphoimidazole and water.

The unusually narrow line widths and the absence of hyperfine structure in the electron spin resonance spectra of the imidazolyl and pyrazolyl radicals shown in Figure 2 are probably due to rapid hydrogen atom exchange. It is likely that imidazole molecules form aggregates in the frozen aqueous sample. If the imidazolyl radical in an aggregate can extract a hydrogen atom from a neighboring imidazole molecule within the relaxation time of its unpaired electron, a narrowing of the electron spin resonance signal similar to that caused by spin exchange may result. It was hoped that by replacing imidazole with the less symmetrical 2,4-dimethylimidazole in the above experiment the aggregation of the base in the frozen sample can be minimized. The observed electron spin resonance of such a system (Figure 2C) indeed exhibits some hyperfine structure. If it can be assumed that hyperfine splittings due to contact interaction of the unpaired electron with the protons of 2,4-dimethylimidazolyl radical are obscured by local anisotropy in the frozen sample, then Figure 2C is consistent with the electron spin resonance spectrum of isolated radicals with two approximately equivalent N atoms superposed on the narrow electron spin resonance signal due to the radicals in molecular aggregates.

A mechanistic scheme for the probable reactions which generate the phosphoimidazolyl radicals is given in Figure 4. Initially when an aqueous solution of hematoporphyrin and imidazole in phosphate buffer was illuminated with yellow light, only imidazolyl radical was formed by the light-driven electron transfer from imidazole to hematoporphyrin. But as the concentration of 1-phosphoimidazole continued to increase during prolonged illumination, the radical **2** may be formed through the direct extraction of an electron from 1-phosphoimidazole (**1**) by the excited hematoporphyrin (HP*). In view of its electron deficiency, we expect **2** to be unusually susceptible to the nucleophilic attack by imidazole and H_2O (or OH^-) to form the radicals **4** and **3**, respectively. The imidazolyl radical **5** may also react directly with $H_2PO_4^-$ and 1-phosphoimidazole to form **3** and **4**, respectively.

If the ^{14}N nuclei in **4** interact approximately similarly with the unpaired electron and if the ^{31}P nucleus (spin = $^1/_2$) interacts much more strongly with the unpaired electron, the electron spin resonance spectrum of **4** should consist of two slightly overlapping nonets, each with relative intensities 1:4:10:16:19:16:10:4:1.

In addition, if we assume that radicals **4** and **5** are of greater stability and hence at considerably higher steady-state concentrations than **2** and **3**, we would expect the frozen mixture to exhibit, upon illumination, an electron spin resonance equivalent to the superposition of the spectra of **4** and **5**. That is, the superposition of a spectrum of two slightly overlapping nonets and the spectrum shown in Figure 2A. Although local anisotropy in the frozen samples prevents us from making reliable assignments of the electron spin resonance line, the observed spectra in Figure 3 are all consistent with this interpretation. It is interesting to note that the hyperfine lines in the electron spin resonance 3B are even more clearly defined than those in spectra 3A and 3C. This last observation is quite consistent with the mechanistic scheme given in Figure 4, since the sample for Figure 3B contains a higher concentration of 1-phosphoimidazole.

These results show that the imidazolyl radical generated by electron transfer can rapidly form a labile P–N bond with inorganic orthophosphate and be subsequently reduced to the energy-rich 1-phosphoimidazole in aqueous solution at pH 7. In the present model system with light-driven electron transfer, the only appreciable overall net reaction is the condensation of inorganic orthophosphate with imidazole to form 1-phosphoimidazole and water. The remarkable cleanness and efficiency of this coupling mechanism suggest its possible application to the coupling of phosphorylation to electron transport in mitochondria and chloroplasts. With the assumption of the ultimate simplicity in nature, we proposed a working hypothesis for mitochondrial oxidative phosphorylation (Wang, 1967) in which the imidazole group of a histidine residue and phosphoester group of a phospholipid (or phosphoprotein) play the respective roles of the imidazole and P_i of the model system. The hypothesis also included a molecular mechanism for respiratory control which recently has gained direct experimental support from work of Wilson and Dutton (1970).

The validity of oxidative phosphorylation mechanisms involving phosphorylated intermediates has often been challenged by investigators of mitochondria-catalyzed isotopic exchange reactions. For example, Boyer and Harrison (1954) discovered that intact mitochondria catalyze the ^{18}O exchange between P_i and H_2O with an efficiency which is independent of electron transport. There seem to be two possible interpretations of this experimental result. (1) The ^{18}O exchange is catalyzed by an ATP-dependent enzyme or system of enzymes similar to the muscle proteins. Consequently the ^{18}O exchange is neither directly dependent upon electron transport, nor a measure of the rate of the subsequent water production reaction. (2) The ^{18}O exchange and the water production are both due to the following catalytic reaction involving a nonphosphorylated energy-rich intermediate ($P_i \cdot ADP$)

$$(P_i \cdot ADP) \overset{\text{enzymes}}{\rightleftharpoons} H_2O + ATP$$

Accordingly, the ^{18}O exchange and the water production are not directly dependent upon electron transport, and oxidative phosphorylation does not involve a phosphorylated intermediate (Boyer, 1967).

Since the phosphorylated intermediate in mitochondrial

oxidative phosphorylation has recently been detected (Cross *et al.*, 1970), it would appear that the second interpretation is not right.

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Effects of Dimethyl Sulfoxide on the Degradation of Ribonucleic Acid Catalyzed by Alkali*

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ABSTRACT: The degradation of RNA by alkali has been measured in media containing Me$_2$SO, a strong nucleic acid denaturant. At 37°, in 0.1 M NaOH, the degradation, measured as the disappearance of acid-precipitable material follows first-order kinetics with a half-life of 23 min (k = 0.03 min^{-1}). Increasing Me$_2$SO concentrations stimulate the degradation rate, until at 60% (v/v) Me$_2$SO the half-life is 1.1 min (k = 0.63 min^{-1}).

Similar kinetic effects are seen following the disappearance of material excluded from Sephadex G-25 (no Me$_2$SO, k = 0.14 min^{-1}; 60% Me$_2$SO, k = 1.39 min^{-1}). The rate enhancement produced by Me$_2$SO is seen between 0.01 and 0.3 M NaOH. Activation enthalpies in 0.1 M NaOH do not differ between 0 and 60% Me$_2$SO (21,200 and 20,700 cal mole^{-1}, respectively). Following the degradation kinetics by means of chromatography on DEAE-Sephadex in the presence of urea, it is seen that in 0.1 M NaOH–60% Me$_2$SO at 55°, in 1 hr more than 96% of RNA has been converted into mononucleotides. This is applicable to the determination of nucleotide composition, where results comparable to other established procedures are obtained. Me$_2$SO also increase the degradation of the dinucleotide UpA, but to a lesser extent than what is seen with polyribonucleotides (k = 0.24 and 0.58 hr^{-1}, for 0 and 60% Me$_2$SO, respectively).

Dimethyl sulfoxide, as well as other aprotic solvents such as formamide, is frequently used as a denaturing agent for nucleic acids (Helmkamp and Ts'o, 1961; Katz and Penman, 1966; Legault-Démare *et al.*, 1967). Although the exact manner by which it acts is not clear, it is generally assumed that its denaturing effectiveness is due to its ability to act as a rather strong acceptor for hydrogen bonding (Lindberg, 1961).

In a different experimental direction, it has been reported that a great number of base-catalyzed reactions, including ester saponification and nucleophilic displacements, are greatly accelerated when performed in media containing Me$_2$SO (Kingsbury, 1964; Roberts, 1965, 1966).

RNA is very sensitive to degradation in alkaline aqueous solutions, but there is no information concerning its sensitivity to alkali in Me$_2$SO–water mixtures. We now wish to report the marked changes in degradation kinetics that occur in such systems, as well as experiments that try to explain the mechanisms involved.

Materials and Methods

The progress of alkali-catalyzed degradation of RNA, measured as the disappearance of acid-precipitable material was as follows. Incubation mixtures, containing NaOH, NaCl when appropriate, and water or Me$_2$SO–water mixtures in a volume of 0.9 ml, were brought to the desired temperature, and the reaction was started by adding 0.1 ml of a solution containing 20 mg/ml of yeast RNA, also equilibrated to the desired temperature. Mixing was made in less than 10 sec. At appropriate intervals 0.1-ml aliquots were taken and added to test tubes containing 0.8 ml of ice-cold 10% perchloric acid–0.25% uranyl acetate; 0.2 ml of 10% beef serum albumin was added as a coprecipitant and the mixture was agitated. After 45 min at 5° the tube contents were filtered through Whatman No. 42 paper. Two-tenth milliliter of the filtrate was

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