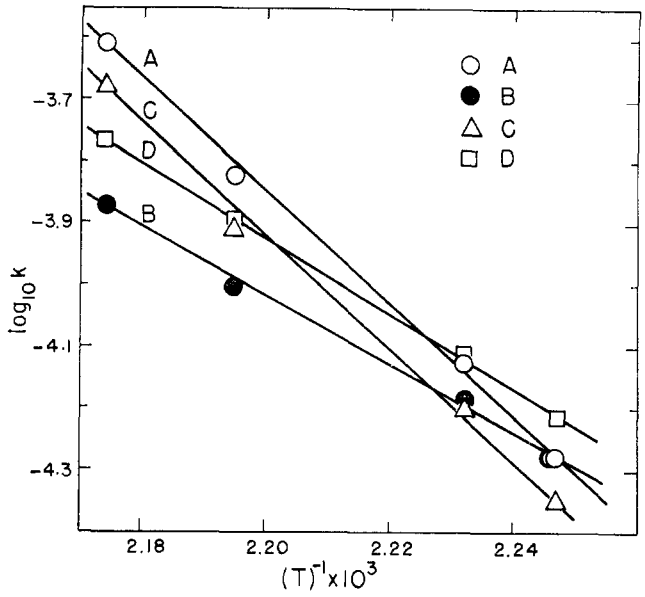


Fig. 1.—Arrhenius plots of first-order rate constants for the internal and external rearrangement of 5-norbornene-2,3-*endo*-dicarboxylic anhydride: A, k_{ext}, uncorrected; *B*, k_{int}, uncorrected; C, k_{ext}, with 15% correction (see text); k_{int}, with 15% correction.

mechanism and dissociation-recombination of addends.[7] The internal route for isomerization may be related to the dissociation–recombination, and hence pertinent to mechanistic representations of the Diels-Alder reaction.[6,7]

We now report evidence which seems to indicate that the two mechanisms for thermal rearrangement of 5-norbornene-2,3-*endo*-dicarboxylic anhydride involve different energy paths.

Rearrangement of the *endo*-anhydride (I) in decalin solution in the presence of tetracyanoethylene gave 2,2,3,3-tetracyanonorborn-5-ene from cyclopentadiene produced by dissociation on the external path(s) as well as *exo*-anhydride from the internal route. The ratio of 2,2,3,3-tetracyanonorborn-5-ene to 5-norbornene-2,3-*exo*-dicarboxylic anhydride was determined by integration of the n.m.r. spectrum of the mixture of these two products and unrearranged *endo*-anhydride and found to be markedly temperature dependent; 1.72 ± 0.05 at 185°, 1.07 ± 0.03 at 174°, 0.63 ± 0.03 at 160°.

A kinetic study of the rearrangement of the *endo*-anhydride to the *exo*-isomer in decalin solution in the absence of tetracyanoethylene led to first-order rate constants for the conversion: $k(\text{sec.}^{-1}) \times 10^5 = 38 \pm 3$ at 187°, 25 ± 2 at 182.5°, 14 ± 1 at 175° and 10.6 ± 1 at 172°.

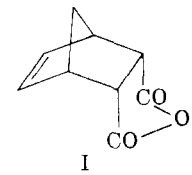
By assuming that tetracyanoethylene reacts much faster than maleic anhydride throughout the temperature range in question[8] and that all the cyclopentadiene–tetracyanoethylene adduct stems from the *endo*-anhydride, calculation gives Arrhenius activation energies and pre-exponential factors for the internal and external processes: $E_{ext} = 42$ kcal. mole^{-1}, $E_{int} = 25$ kcal. mole^{-1}, $A_{ext} = 10^{16}$ sec.$^{-1}$, A_{int} $10^{8.8}$ sec.$^{-1}$.

(7) J. A. Berson and R. D. Reynolds, *J. Am. Chem. Soc.*, **77**, 4434 (1955); J. A. Berson, R. D. Reynolds and W. M. Jones, *ibid.*, **78**, 6049 (1956).

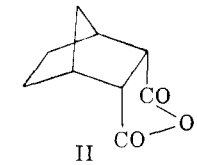
(8) (a) *Cf.* J. A. Berson and W. A. Mueller, *ibid.*, **83**, 4940 (1961); (b) The possibility that any significant amount of *exo*-maleic anhydride adduct arises by dissociation of the tetracyanoethylene adduct after the latter is formed, was ruled out by control experiments. At 195° in decalin, about 7% of maleic anhydride adduct was formed by heating the tetracyanoethylene adduct in the presence of excess maleic anhydride for 35 min. When the tetracyanoethylene adduct was heated with tetracyanoethylene and maleic anhydride in 1.2 molar proportions, the yield of maleic anhydride adduct dropped to 2%.

The second assumption ignores the fact that some of the cyclopentadiene–tetracyanoethylene adduct stems from the *exo*-anhydride first formed through the internal mechanism. Since the kinetic data for rearrangement in the absence of tetracyanoethylene exhibited first-order behavior until 40–50% conversion and the rearrangements in the presence of tetracyanoethylene were carried to 50–70% conversion, the proportion of 2,2,3,3-tetracyanonorborn-5-ene formed from the *exo*-anhydride cannot be large. This uncertainty prohibits calculation of reliable values for entropies and energies of activation, but it does not obscure the basic results. For instance, if 15% of the 2,2,3,3-tetracyanonorborn-5-ene came from the *exo*-anhydride, the data would lead to $E_{ext} = 42$ kcal. mole^{-1}, $E_{int} = 28$ kcal. mole^{-1}, $A_{ext} = 10^{16}$ sec.$^{-1}$, $A_{int} = 10^{9.7}$ sec.$^{-1}$. With or without a correction for this second external path, the calculated first-order rate constants for the internal and external rearrangement of the *endo*-anhydride give good straight lines in Arrhenius plots (Fig. 1) and the estimated kinetic parameters for the two processes are quite dissimilar.

Further, 2,3-*endo*-norbornanedicarboxylic anhydride (II) was converted to the extent of 22 ± 2% to its *exo*-isomer when heated to 250° for 24 hours. Since the ring system of I is likely to be more strained than that of II, it should be labile to rearrangement under relatively less severe conditions.



I



II

In view of the widely different energetic parameters characterizing the two processes, and of the thermal rearrangement of 2,3-*endo*-norbornanedicarboxylic anhydride to its *exo*-isomer at 250°, we regard as remote the possibility that the two pathways for thermal isomerization of I are closely related.

This work suggests that kinetic studies with systems capable of interconversions similar to those shown by the 1-hydroxydicyclopentadienes may clarify the relationship of these rearrangements with Cope rearrangements[2-5] and Diels–Alder reactions.

More detailed investigations of mechanisms of the thermal isomerization of I to its *exo*-isomer will be reported later.

We thank Professor J. A. Berson for his helpful interest and suggestions regarding this study.

(9) National Science Foundation Predoctoral Fellow, 1960–1962; Department of Chemistry, University of Illinois, Urbana, Illinois.

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RECEIVED NOVEMBER 15, 1962

PHOTOCHEMICAL REDUCTION OF NITRATE CATALYZED BY MOLYBDENUM(VI) AND FLAVIN MONONUCLEOTIDE

Sir:

The biological reduction of nitrate to nitrite, catalyzed by the molybdoflavoenzyme nitrate reductase, has been indicated to proceed through the following sequence of electron transfers[1]

$$\text{TPNH} \longrightarrow \overset{\text{FAD}}{\underset{\text{FMN}}{\text{or}}} \longrightarrow \text{Mo(VI)} \longrightarrow \text{NO}_3^-$$

(1) D. J. D. Nicholas and A. Nason, *J. Biol. Chem.*, **211**, 183 (1954).

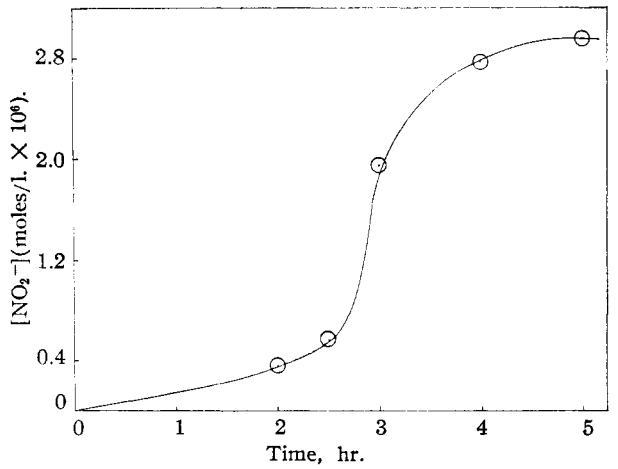

Fig. 1.—Formation of nitrite with time: initial concentrations: $[NO_3^-] = 0.128\ M$, FMN = $9.00 \times 10^{-3}\ M$, Mo(VI) = $0.119\ M$; pH 6.3 (phosphate buffer).

Stoy reported that a system consisting of nitrate reductase, riboflavin and EDTA reduced nitrate when irradiated with blue light for short periods.[2] In this case the light apparently reduced the riboflavin, which then served as reducing agent. Only traces of nitrite were formed in the absence of the enzyme.

We wish to report the anaerobic, non-enzymatic reduction of nitrate by a model system consisting of FMN and Mo(VI) using visible light. We found that in the absence of FMN or Mo(VI), or without irradiation, no reduction was observed. In order to prevent the known direct reduction of nitrate by ultraviolet light of wave length shorter than 320 mu,[3] a 100-watt tungsten lamp equipped with a Corning Glass filter, CS 3-75, which cuts off all wave lengths shorter than 375 mu was used as the light source. The method of Snell and Snell, as modified by Shinn, was used to determine nitrite.[4] All reactions were run in phosphate buffer at pH 6.3.

Figure 1 illustrates the formation of nitrite with time. It is apparent that there is a period before any nitrite is detected. This may be due to a relatively slow reaction of photoreduced FMN with Mo(VI) to produce Mo(V), which subsequently reacts with nitrate.

It is well known that photoreduced FMN will react directly with a variety of reducible species. In this regard, the reaction reported here is of considerable interest since, although the oxidation–reduction potential is favorable (E_0' at pH 6.3 for the reaction $FMNH_2 + NO_3^- \rightarrow NO_2^- + FMN + H_2O$, is +0.75 volt), the expected direct reduction of nitrate by photoreduced FMN does not occur, under the conditions used, in the absence of Mo(VI). It would thus appear that Mo(VI) acts as a catalyst, probably reacting with reduced FMN to form Mo(V) which in turn reduces nitrate, regenerating Mo(VI). Direct spectrophotometric evidence for a reaction between photoreduced FMN and Mo(VI) is difficult to obtain, since FMN and Mo(V) absorb in the same region of the spectrum and photolysis produces changes in the spectrum of FMN. Williams has reported a slow reaction between chemically reduced riboflavin and Mo(VI) to produce Mo(V)[5] and polarographic evidence obtained in this laboratory indicates that when FMN has been reduced photochemically in the presence of Mo(VI), the oxidation wave for the photoreduced species slowly disappears, reforming the

(2) V. Stoy, *Biochim. Biophys. Acta*, **21**, 395 (1956).
(3) R. Cultrera and G. Ferrari, *Ann. chim. (Rome)*, **47**, 1321 (1957).
(4) M. B. Shinn, *Ind. Eng. Chem., Anal. Ed.*, **13**, 33 (1941).
(5) R. J. P. Williams, "Advances In The Chemistry of The Coordination Compounds, Proceedings of the Sixth International Conference on Coordination Chemistry," S. Kirschner, ed., The Macmillian Co., New York, 1961, p. 73.

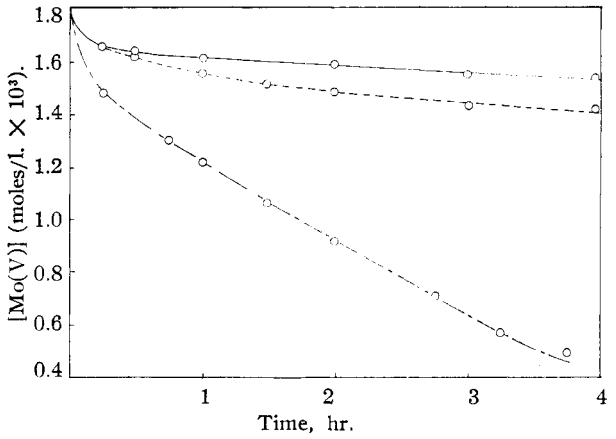

Fig. 2.—Reaction of Mo(V) with NO_3^- and NO_2^-: Mo(V) concentration determined by following its absorbance at 289 mμ: initial concentrations: —— $[NO_3^-] = 2.04 \times 10^{-2}\ M$, [Mo(V)] = $1.80 \times 10^{-3}\ M$; – – – $[NO_3^-] = 2.04 \times 10^{-2}\ M$, [MO(V)] = $1.80 \times 10^{-3}\ M$, [FMN] = $5.00 \times 10^{-4}\ M$; – – – $[NO_2^-] = 1.90 \times 10^{-2}\ M$, Mo(V) = $1.80 \times 10^{-3}\ M$, pH 6.3 (phosphate buffer).

reduction wave characteristic of FMN, while in the absence of Mo(VI) the oxidation wave remains constant. Further evidence for Mo(V) as an intermediate in the reduction was obtained by studying its reaction with nitrate (Fig. 2). It is seen that it reacts slowly, but that the rate is increased by the addition of FMN. Finally, after a solution of FMN, nitrate and Mo(VI) has been photolyzed for some time (1–2 hours) and opened to the atmosphere, molybdenum blue is formed upon acidification, indicating the presence of Mo(V).

The rate of formation of nitrite (Fig. 1) slows down with time and its concentration apparently approaches a steady state. This is very likely due to a subsequent reaction of nitrite with Mo(V) to produce nitric oxide, and perhaps other compounds of lower oxidation state. Figure 2 illustrates the reaction of Mo(V) with nitrite. A sample of gas evolved by this reaction mixture was analyzed on a gas chromatograph and shown to be almost entirely nitric oxide. No attempt was made to determine the amount of gas produced. It should be noted that the reaction of Mo(V) with nitrite is somewhat faster than with nitrate. The rather low level of nitrite detected is accounted for by this subsequent reaction.

When the reduction was attempted aerobically only traces of nitrite were detected. This is most likely due to the rapid reaction of photoreduced FMN with oxygen,[6] which precludes the formation of Mo(V).

It appears that the function of FMN in the reduction is two-fold: (1) it serves, when photoreduced, as a reductant for Mo(VI) and (2) it acts as a catalyst in the reaction of Mo(V) with nitrate. It also has been reported that FMN acts as a catalyst in the oxidation of Mo(V) by atmospheric oxygen under similar conditions.[7] This catalytic effect is no doubt an example of Shaffer's "Equivalence Change Principle."[8]

It is clear that the system reported here functions in a manner similar to nitrate reductase, and may serve as a model for this enzyme. These reactions are proposed for the reduction

$$FMN \xrightarrow{h\nu} FMNH^9$$
$$FMNH + Mo(VI) \longrightarrow FMN + LC + Mo(V)$$
$$2Mo(V) + NO_3^- \longrightarrow 2Mo(VI) + NO_2^-$$
$$Mo(V) + NO_2^- \longrightarrow Mo(VI) + NO$$

(6) G. Strauss and W. J. Nickerson, *J. Am. Chem. Soc.*, **83**, 3187 (1961).
(7) J. T. Spence and J. Tocatlian, *ibid.*, **83**, 816 (1961).
(8) P. A. Shaffer, *J. Phys. Chem.*, **40**, 1021 (1936).
(9) The exact nature of the photoreduced FMN is not known at present and is indicated simply as FMNH without implying anything concerning

A detailed study of the kinetics and mechanism of each of these reactions is now underway in this laboratory and will be reported later.

Acknowledgment.—Thanks are expressed to The James G. Boswell Foundation (Stanford Research Institute) and to The Public Health Service (Grant GM 08347-02, Division of General Medical Sciences) for financial support.

its structure [G. Oster, J. S. Bellin and B. Holmstrom, *Experientia*, **18**, 249 (1962)]. Polarographic studies in this laboratory indicate both FMN and lumichrome (LC) are formed upon reoxidation of photoreduced FMN.

DEPARTMENT OF CHEMISTRY
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RECEIVED SEPTEMBER 18, 1962

THE SEPARATION OF GUANOSINE OLIGONUCLEOTIDES: USE OF UREA TO AVOID AGGREGATE FORMATION

Sir:

Separation of guanine-rich oligonucleotides by usual methods is difficult and unreproducible, presumably because of aggregation of such compounds. We wish to report that a satisfactory separation can be carried out by DEAE-cellulose chromatography with the addition of 7 *M* urea, as described by Tomlinson and Tener[1] for the separation of ribonuclease digests of nucleic acids.

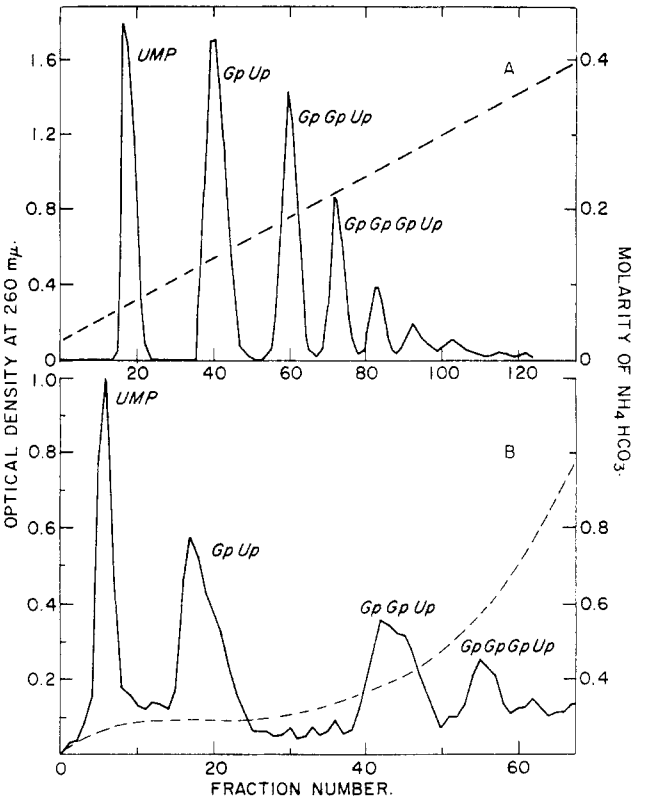

Fig. 1.—A. Separation of a pancreatic ribonuclease digest of poly GU (570 O.D. units at 260 mμ) adsorbed on a 12.8 × 2 DEAE-cellulose–bicarbonate column, eluted with a linear gradient. The mixing chamber contained 750 ml. of 0.03 *M* NH_4HCO_3 in 7 *M* urea, the reservoir 750 ml. 0.5 *M* NH_4HCO_3 in 7 *M* urea, both at pH 8.6. Samples of 8 ml. were collected at a flow rate of 2 ml./min. B. Similar to (A), 184 O.D. units at 260 mμ adsorbed on a 10 × 1 cm. column, eluted with a 600 ml. non-linear gradient[7] of NH_4HCO_3, pH 8.6, as shown, collecting 10 ml. samples at a flow rate of 0.75 ml./min.

It has been shown[2] recently that deoxyguanosine oligonucleotides possess a secondary structure and are capable of forming aggregates. We have independent evidence that points to secondary structure in guanosine oligonucleotides. (1) A mixture of guanine-containing oligonucleotides of average chain length equal to 6 was unusually resistant to Takadiastase ribonuclease T_1[3] and pork liver nuclease.[4] A preliminary heating of the substrate resulted in somewhat more rapid hydrolysis, but the rates still were very slow. (2) Removal of terminal phosphate by *E. coli* alkaline phosphatase[5] was extremely slow and incomplete for GpGpGpGp, GpGpGpUp and homologs of higher molecular weight. (3) Paper chromatograms of pancreatic ribonuclease digests of poly GU[6] showed the presence of 3′-UMP, GpUp, GpGpUp and GpGpGpUp as discrete bands, but nothing else migrated from the origin even after several days development in the tank. Material at the origin was hydrolyzed and found to have a ratio guanine/UMP of 4.2/1. This indicates that oligonucleotides containing uracil did not display the expected mobility in the solvent system. Further, when GpGpUp, GpGp, GpG or larger homologs of these were re-chromatographed, over a third of the material remained at the origin.

The use of 7 *M* urea with the DEAE-cellulose column appears to prevent aggregation. With urea (Fig. 1), a simple linear gradient gave sharper peaks, better resolution and better return of optical density to the baseline between peaks than was obtained without urea and using a gradient deliberately flattened[7] to allow maximum resolution of the first four peaks.

Striking differences also were noted when single compounds were chromatographed on DEAE. Thus, GpGpUp (40.2 O.D. units at 260 mμ) was applied in 7 *M* urea to a DEAE-bicarbonate column (2.8 cm.^{3}) and eluted, with a linear gradient of NH_4HCO_3 in 7 *M* urea, as a single peak. Recovery was 40.8 O.D. units (101%). By contrast, chromatography of GpGpUp in the absence of urea led to recovery of only 65% of the material in the expected elution position, and even eluting the column with high salt did not completely remove the remainder.

Similarly, a digest containing a mixture of guanine oligonucleotides with 2′ (or 3′) phosphate end groups, eluted in the presence of urea, yielded a series of sharp peaks, of which the first five accounted for over 90% of the starting material. Chromatography as usually carried out with DEAE[7] or Dowex-1-Cl^-, 2× cross-linked,[8] was quite unsatisfactory, since even a compound as simple as GpG could not be recovered quantitatively.

(1) R. V. Tomlinson and G. M. Tener, *J. Am. Chem. Soc.*, **84**, 2644 (1962).

(2) R. K. Ralph, W. J. Connors and H. G. Khorana, *ibid.*, **84**, 2265 (1962).

(3) K. Sato-Asano and F. Egami, *J. Biochem.* (Japan), **44**, 753 (1957).

(4) M. N. Lipsett, L. A. Heppel and W. E. Razzell, unpublished data.

(5) A. Torriani, *Biochim. et Biophys. Acta*, **38**, 460 (1960).

(6) Poly GU is a random copolymer of uridylic and guanylic acids.

(7) Using the varigrad of E. A. Peterson and H. A. Sober, *J. Am. Chem. Soc.*, **78**, 751 (1956); *Anal. Chem.*, **31**, 857 (1959).

(8) E. Volkin and W. E. Cohn, *J. Biol. Chem.*, **205**, 767 (1953).

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RECEIVED NOVEMBER 12, 1962

CONCERNING THE STRUCTURE OF THE GRIGNARD REAGENT

Sir:

Although the Grignard reagent has been in use for over sixty years, considerable confusion exists as to the precise nature of this reagent in ether solution. The composition of the Grignard reagent has been represented most often by the equilibria[1]

$$2RMgX \rightleftharpoons R_2Mg + MgX_2 \rightleftharpoons R_2Mg \cdot MgX_2$$

(1) M. S. Kharasch and O. Reinmuth, "Grignard Reactions of Nonmetallic Substances," Prentice-Hall, Inc., New York, N. Y., 1954.