O-Carbamyl-N-carbobenzoxy-L-serine Benzyl Ester.—The intermediate, N-carbobenzoxy-L-serine benzyl ester, was prepared by the procedure described above for the DL-isomer. Baer and Maurukas[11] also prepared this intermediate by a different procedure.

Reaction of 1.0 g. of N-carbobenzoxy-L-serine benzyl ester, in the same general procedure as described for the DL-isomer, with phosgene followed by treatment with ammonium hydroxide gave 0.69 g. (61% of theory) of the benzyl ester of O-carbamyl-N-carbobenzoxy-L-serine, m.p. 104–106°.

Anal. Calcd. for $C_{19}H_{20}N_2O_6$: N, 7.52. Found: N, 7.68.

O-Carbamyl-L-serine.—A solution of 0.60 g. of O-carbamyl-N-carbobenzoxy-L-serine benzyl ester dissolved in 100 ml. of 50% dioxane, in the presence of 200 mg. of palladium black catalyst, was treated with hydrogen at room temperature and atmospheric pressure for 4 hr. The catalyst was filtered, washed with hot water and the combined filtrates were taken to dryness under reduced pressure. The residue was then recrystallized from alcohol–water to yield 85 mg. (36% of theory) of white needles, m.p. 206–209° dec.; $[\alpha]_D$ + 19.9° (*c* 2 in 1 *N* HCl).

Anal. Calcd. for $C_4H_8N_2O_4$: C, 32.44; H, 5.45; N, 18.93. Found: C, 32.70; H, 5.59; N, 18.73.

AUSTIN, TEXAS

[CONTRIBUTION FROM THE WESTERN UTILIZATION RESEARCH BRANCH][1]

Cross-linking of Bovine Plasma Albumin and Wool Keratin

BY J. E. MOORE AND W. H. WARD

RECEIVED OCTOBER 14, 1955

N-Ethylmaleimide is recognized as a specific additive reagent for thiol groups[2] with the advantages of mild reaction conditions and absence of by-products. N-Substituted bis-maleimides therefore have been studied as possible primary valence cross-linking reagents for proteins having reactive thiol groups. An instance of cross-linking by such a reagent has been shown in the reaction of bovine plasma albumin with N,N′-(1,3-phenylene)-bis-maleimide. Effects of treating reduced wool with N-phenylmaleimide and N,N′-bis-maleimides are described and evidence for cross-linking evaluated.

Introduction

Blood plasma albumin commonly includes a component, mercaptalbumin, having a single reactive thiol group per molecule.[3] The remainder of the albumin is closely similar in molecular weight, composition and other properties but contains no reactive thiol groups.

Mercaptalbumin forms about two-thirds of the albumins as usually isolated, but the reactive thiol content is affected by exposure of the sample to reducing media or prolonged storage. Because of this single reactive thiol group, bovine plasma albumin was selected as an appropriate protein for testing the cross-linking properties of bis-maleimides. The ultracentrifuge was used as a convenient instrument for demonstrating cross-linking and estimating the amount of "dimer" formed, since the centrifugal behavior of "dimerized" plasma albumin cross-linked by means of mercury through the thiol groups is known.[4]

Bis-maleimides have also been tested as cross-linking agents for reduced wool because of the desirability, in some applications, of replacing the disulfide cross-links by others more resistant to alkali, oxidation and reduction. Because evidence of cross-linking in wool is mainly indirect and ambiguous, comparative measurements of mechanical properties and resistance to chemical degradation have been made with fibers treated with difunctional and monofunctional reagents. These measurements have been supplemented by cystine analyses, stoichiometry of reagent uptake, and chromatographic demonstration of the expected cysteine derivative to provide as conclusive evidence as possible for the covalent cross-linking of wool by bis-maleimides.

Materials and Methods

Preparation of Cross-linking Agents.—N,N′-(1,2-Phenylene)-bis-maleamic acid was prepared by the directions given by Schönberg and Mustafa.[5] This method was also found satisfactory for the unreported meta isomer which decomposed near 216° after crystallization from ethanol–dimethylformamide solution.

Anal. Calcd. for $C_{14}H_{12}N_2O_6$: C, 55.5; H, 4.0; N, 9.2. Found: C, 55.5; H, 4.4; N, 9.2.

N-Phenylmaleimide was prepared by the method of Searle.[6] The bis-maleamic acids were converted to bis-maleimides by the same procedure. The unreported N,N′-(1,2-phenylene)-bis-maleimide (OPBM) melted at 243–244° after crystallization from dimethylformamide as fine white crystals.

Anal. Calcd. for $C_{14}H_8N_2O_4$: C, 62.7; H, 3.0; N, 10.5. Found: C, 62.6; H, 3.0; N, 10.5.

Plasma Albumin.—Armour's Bovine Plasma Albumin (Control N 128-175)[7] was used. Air-dry samples of 1.1 g. corresponding approximately to 1.0 g. dry weight were dissolved in 3.0 ml. of distilled water, each, in 12-ml. centrifuge tubes. The *p*H was adjusted to 8.0 in most experiments with a small amount of 50% NaOH or 28% NH_3. The quantities of $HgCl_2$ (mol. wt. 271.5) and N,N′-(1,3-phenylene)-bis-maleimide (MPBM) (mol. wt. 268.2) calculated to be equivalent to $^2/_3$ g. of mercaptalbumin (mol. wt. = 65,000) are 1.39 and 1.38 mg. Approximately this amount or $^1/_2$ of this amount was added as shown in Table I. The result of a preliminary experiment with excess N,N′-(1,2-phenylene)-bis-maleimide (OPBM) is also shown. Mercuric chloride was added as a solution in 1.0 ml. of distilled water, the organic reagents as dry crystalline powders. The tubes were stoppered, placed in the cold room at 7° and mechanically inverted at intervals of a few seconds. The bis-maleimides disappeared during reaction but remained undissolved when in excess. Samples without the cross-linking reagents were treated in the same way at the same time for use as controls.

(1) Agricultural Research Service, U. S. Department of Agriculture, Albany, 10, California. Presented at the ACS meeting, Minneapolis, Sept. 11–16, 1955.

(2) T. C. Tsao and K. Bailey, *Biochem. Biophys. Acta*, **11**, 102 (1953).

(3) W. L. Hughes, in H. Neurath and K. Bailey, "The Proteins," Vol. II, Part B, Academic Press, New York, N. Y., 1954, Chapter 21.

(4) W. L. Hughes, Jr., THIS JOURNAL, **69**, 1836 (1947).

(5) A. Schönberg and A. Mustafa, *J. Chem. Soc.*, 654 (1943).

(6) W. E. Searle (assigned to E. I. du Pont de Nemours and Co.), U. S. Patent 2,444,536 (1948).

(7) Mention of a specific product does not imply endorsement by the U. S. Government.

TABLE I[a]

SEDIMENTATION STUDIES OF CROSS-LINKING OF BOVINE PLASMA ALBUMIN

Analysis	Reagent	pH	Temp.	Sedimentation constants Obsd.	Standard	Proportion of "dimer," %	Remarks
628u	None	5.39	25.0	4.60	4.42		
				6.77	6.49	Trace	Proportion not measured
640u	None	(8)	26.2	4.44	4.12	5	Sedimentation rate of "dimer" not
641u	None	8.40	25.9	4.22	4.04	4	measured
639u	0.6 mg. $HgCl_2$	7.70	24.3	4.54	4.37		
				6.64	6.29	25	
639	1.3 mg. $HgCl_2$	7.75	24.3	4.44	4.26		
				6.49	6.24	46	
638u	0.6 mg. MPBM	5.50	23.4	4.33	4.25		
				6.29	6.17	15	
638	1.25 mg. MPBM	5.50	23.4	4.36	4.28		
				6.32	6.20	23	
640	1.2 mg. MPBM	(8)	26.2	4.66	4.32		Measured 4.5 hr., after mixing
				6.75	6.14	35	
641	1.2 mg. MPBM	8.07	25.9	4.56	4.17		Measured 24 hr. after mixing
				6.59	6.04	40	
642	MPBM	(8)	23.6	4.34	4.23		Measured 20 hr., after adding 0.85 mg.
				6.16	6.00	40	excess reagent to preceding sample
643	MPBM	(8)	25.3	4.75	4.47		Preceding sample analyzed at 1/3 the
				7.00	6.58	41	protein concn.
628	OPBM	5.37	25.0	4.61	4.42		
				6.76	6.49	Trace	Reagent in excess. No stirring (More than in control)

[a] Reactions were carried out at 20 to 25% protein in distilled water with adjusted *p*H. Analyses were made at 1% protein (1/3% for 643) at the *p*H and average temperature shown. The samples were diluted to give 0.2 *N* KNO_3 (NaCl for 628 and 628u) just before analysis. Except for 628 and 628u, samples were stirred by inverting end-for-end about once per second. Reagents were mixed at room temperature, then transferred, together with control samples without the bis-maleimide or $HgCl_2$, to a cold room at 7°. The weights given were added per gram albumin. The larger amounts specified approximate the amounts calculated to cross-link 2/3 of the albumin (mol. wt. = 65,000) present, in accordance with the results of Hughes. Sedimentation constants are in Svedberg units and are given both as observed and referred to the medium: water at 20°. The proportion of dimer was estimated from planimeter measurements of the sedimenting boundary records.

A Spinco Model E analytical ultracentrifuge[7] was used at the maximum operating speed, 59,780 r.p.m. (centrifugal force about 250,000 × *g*). Prismatic and regular cells were used so that treated and control samples—or samples with two levels of treatment—could be analyzed at the same time. Samples analyzed in the cell with the prismatic window are designated "u" in Table I.

The concentrated albumin samples were diluted with aqueous salt solution to give 0.2 ionic strength, either with NaCl or, in most instances KNO_3, for centrifugal analysis.

Preparation and Treatment of Reduced Wool.—An 8 1/4 oz. Botany style 404 white flannel wool, thoroughly solvent-extracted and divided into squares of approximately two grams in weight, was used. Test fibers calibrated with respect to stress-strain behavior and with the ends mounted on cellophane tabs were sewed into the flannel.

The wool samples were reduced by immersing in 0.4 *M* 2-mercaptoethanol for one hour at 50°; 30 ml. of solution per gram of wool was used throughout the experiment with 0.1% Triton X-100, a non-ionic detergent, added to facilitate wetting. The flannels containing test fibers were then washed for one hour under running water.

After reduction, the wool samples were immediately transferred to a suspension of the maleimide in 0.1 *M* sodium borate buffer at *p*H 8.0.

One millimole of the bis-maleimide or 2.0 mmoles of the N-phenylmaleimide in 30 ml. of buffer containing 0.2% wetting agent was generally used per g. of wool.

The closed container was then shaken at room temperature till the nitroprusside test in 8 *M* guanidine hydrochloride was negative. The time of shaking varied from one hour, for the more soluble N-phenylmaleimide, to over 30 hours, for the very insoluble bis-maleimides.

The wool samples were then washed for 30 minutes in running water, equilibrated with *p*H 5.5 0.1 *M* sodium citrate buffer for one hour and finally washed in running water for several hours.

Methods of Testing.—The following tests were employed as evidence of cross-linking in wool:

1. **Alkali solubility** was determined by immersing the flannel in 0.1 *N* NaOH for one hour at 65° according to the method of Harris and Smith.[8] Untreated flannel gave a value of 10.8%.

2. **Cystine analyses** were made by the Shinohara[9] method after hydrolysis for five hours in 5 *N* HCl at 125° in a sealed tube.

3. The 30% index,[10] defined as the ratio of the energy required to stretch the fiber 30% of its length in water before and after treatment, was determined on the calibrated fibers sewed into the flannel. These values were obtained with a constant rate of loading on the apparatus described by Preusser and co-workers[11] and are the results of at least four determinations.

4. **Degree of supercontraction**[12] was obtained on single fibers exposed to 5% $NaHSO_3$ at 100° for one hour.

5. **Acid solubility** was determined by immersing 400 mg. of the flannel in 4 *N* HCl for one hour at 65° according to the method adopted by Zahn and Würz.[13] Untreated flannel gave a value near 8% under these conditions.

6. **Solubility in ammonia after treatment with peracetic acid** was carried out according to the directions of Alexander, *et al.*[14] Untreated flannel, shaken vigorously at intervals was soluble to the extent of about 90% as reported.

7. **Two-dimensional ascending paper chromatograms** were made of hydrolyzates of the treated and untreated

(8) M. Harris and A. L. Smith, *J. Research Natl. Bur. Standards*, **17**, 577 (1936).

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(11) H. M. Preusser, R. A. O'Connell, A. M. Yeiser and H. P. Lundgren, *Textile Res. J.*, **24**, 118 (1954).

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(13) H. Zahn and A. Würz, *J. Textile Inst. (Proc. Sec.)*, **45**, 88 (1954).

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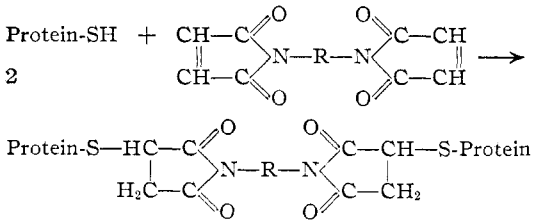
flannels. The solvent system in the first dimension consisted by volume of methanol, water and pyridine, 80/20/4. The second solvent system consisted by volume of phenol and water 35/10 containing 0.04% 8-hydroxyquinoline as preservative. A small beaker of 0.3 *M* NH_4OH was placed in the chamber to facilitate movement of the basic amino acids. Schleicher and Schuell No. 589 Blue Ribbon filter paper was found satisfactory.

Cross-linking of Plasma Albumin.—Results of sedimentation analyses of plasma albumin treated with $HgCl_2$ or the bis-maleimides are given in Table I. These may be summarized as follows.

1. The untreated plasma albumin had observable traces of material, about 5% of the total, sedimenting with the rate of the "dimer."
2. "Dimerization" by $HgCl_2$, as described by Hughes, was reproduced. The maximum dimerization noted with this reagent was 46%. A ninefold excess of the reagent left only "monomer," except for a trace approximately equal to the trace of "dimer" initially present.
3. Treatment with MPBM at levels of 0.60, 1.20 and 1.25 mg. per g. protein produced "dimer" boundaries comprizing, respectively, 15, 35 to 40, and 23% of the total area. Correction of these areas for the effect of dilution with distance sedimented in the sector-shaped cells was found to be unimportant.
4. Sedimentation experiments made 5 and 25 hours after adding MPBM showed little difference in the rates of movement but an increase of 5% in the relative area of the "dimer" boundary. Added excess of MPBM, an additional 0.85 mg., with further shaking for 20 hours made no further change. The additional reagent did not dissolve.
5. The *relative* areas were not affected by the protein concentration during analysis.

Discussion

The near identity of sedimentation constants of plasma albumin components treated with $HgCl_2$ and MPBM is taken to confirm cross-linking ("dimerization") by the latter reagent. However, the dimerization by MPBM is not reversed by dilution or by excess reagent, at least after the "dimer" is formed. This shows that the expected thioether bond formed by equation 1 is not dissociable under these conditions.



A cross-linking reaction of this character may be formulated as proceeding in two steps: (1) A + B → A-B; (2) A + A-B → A-B-A. In this scheme, A represents the monofunctional mercaptalbumin, B the difunctional cross-linking reagent. A-B-A is the cross-linked "dimer." The intermediate product A-B has approximately the same mass as A and cannot be distinguished from it by direct observation in the ultracentrifuge. It would be expected that excess B added immediately to A could produce an excess of A-B, leaving a deficiency of A needed to complete formation of the cross-linked product. This possibility requires not only an available excess of B, but also depends on the relative rates of the two reactions.

In our case, cross-linking is favored by the low solubility of the organic reagents, ensuring that the actual concentration of B will be low throughout the reaction even though the potential supply is in excess. Nevertheless, the negative results of the experiment with OPBM and irregularities in the relation of the areas to reagent added make further experiments of interest to test how the proportion of "dimer" formed in our experiments may be affected by initial excess of reagent, the structure of the reagent, or other experimental conditions. If it can be shown that excess reagent has no effect, a direct means is available for showing the reactive thiol content (accessible to the reagent) of monofunctional -SH containing proteins. If the centrifugal areas are dependent on excess reagent, the general method can still be usefully applied to study this reaction. In either case the equivalent weight and the reactive fraction of the protein can be calculated from the amount of "dimer" formed by submaximal or just maximal amounts of reagent relative to total protein as estimated from the boundary areas. The thiol content of plasma albumin preparations has been reported to vary depending on the history of the sample and experimental procedures used.[15] Our results suggest that our sample had less available mercaptalbumin than observed by either Boyer[14] or Hughes.[2] An alternative explanation may be that steric effects hinder reaction of maleimides with thiol groups.[1,16]

Table II gives the results of tests applied to reduced wools treated with maleimides. The long period of shaking (30 hours for MPBM) needed to eliminate the nitroprusside test with reduced wool may be due to the low solubility of the bis-maleimides taken with slowness of diffusion into the fiber. Evidence for these beliefs is given by the following observations. Nitroprusside in aqueous Na_2CO_3 solution gives a negative test quite early in the course of the reaction. However, if at this time nitroprusside in 8 *M* guanidine hydrochloride is applied, a dull red gradually becomes evident after several minutes, indicating that only the more accessible thiol groups have reacted.

TABLE II

RESULTS OF SELECTED TESTS ON REDUCED WOOL TREATED WITH MALEIMIDES

Tests	Treatment: N-Phenylmaleimide	MPBM	OPBM	Buffer alone
Uptake, %	8.36	5.52	4.15	0
30% Index[a]	0.81	0.88	0.88	0.77
Cystine, %	5.71	6.52	7.50	11.4
Supercontraction, %	22	0.3	0.8	28
Alkali solubility, %	24.4	6.9	7.6	25.3
Acid solubility, %	12.8	6.4	7.0	13.0
Peracetic acid–NH_3 solubility, %	73	44	56	89

[a] The 30% index of the reduced wool was initially 0.67 in all cases.

Treatment with any of the three maleimides raised the 30% index, the bis-maleimides being the most effective. Table II also shows that even shaking in buffer had this effect, although to a lesser degree.

Boiling bisulfite affects normal wool fiber by breaking the disulfide linkages and hydrogen bonds, permitting the fiber to shorten. This supercontrac-

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(16) V. M. Ingram, *Biochem. J.*, **59**, 653 (1955).

tion is strikingly prevented by treating reduced wool with the difunctional maleimides, while the monofunctional derivatives had little effect.

The solubility of the flannel in alkali is quite markedly reduced after treatment with the difunctional maleimides, indicative of stable cross-links between polypeptide chains. Reduced flannel treated with buffer alone or with N-phenylmaleimide was much more soluble.

Peracetic acid attacks the disulfide bonds of wool. Subsequent treatment with dilute NH_3 normally brings about solution of about 90% of the fiber. This amount was greatly decreased by the difunctional maleimides, but to a much lesser extent by the monofunctional reagent.

From the cystine values given, it is possible to calculate the expected uptake of bis-maleimide with or without cross-linking, that this, assuming either that both double bonds react with a thiol group or that only one on a single difunctional molecule reacts. Table III gives such calculations. The amount of combined N-phenylmaleimide agrees closely with the observed decrease in cystine content. Similarly, amounts of the combined bis-maleimides are close to the values required by the decrease in cystine only if it is assumed that both double bonds have reacted.

TABLE III

RELATIONSHIP OF COMBINED REAGENT TO CYSTINE CONTENT

Compound	Reacted cystine, %[a]	Assumed mode of reaction	Theor. reagent uptake (calcd. from cystine data) %	Reagent uptake found, %
MPBM	4.88	Cross-linking	5.45	5.52
OPBM	3.90	Cross-linking	4.35	4.32
N-Phenylmaleimide	5.69	No cross-linking	8.20	8.36

[a] Initial cystine content minus cystine content after treatment.

A more direct method of demonstrating cross-linking consists of finding specific cross-linked amino acid derivatives in a hydrolysate of the treated wool. In this instance both the mono- and the difunctional maleimides yield the same amino acid, 2-amino-2-carboxyethylmercaptosuccinic acid, because the imide bond is not stable to acid hydrolysis. It is important, however, to examine the hydrolysates to show that the indicated reaction has indeed taken place and that no unexpected transformations are yielding misleading results. Figure 1 shows a typical chromatogram of a hydrolysate of wool treated with any of the three substituted maleimides discussed. The expected amino acid was synthesized and its position on the chromatogram compared with that of the new spot found in the treated hydrolysates. This comparison was made by chromatographing the amino acid both alone and in a mixture with a normal hydrolysate. The new spot is indicated by formula. No other differences were noted from a qualitative examination of normal and treated hydrolysates.

All of the tests applied to reduced wools treated with bis-maleimides give results that would be ex-

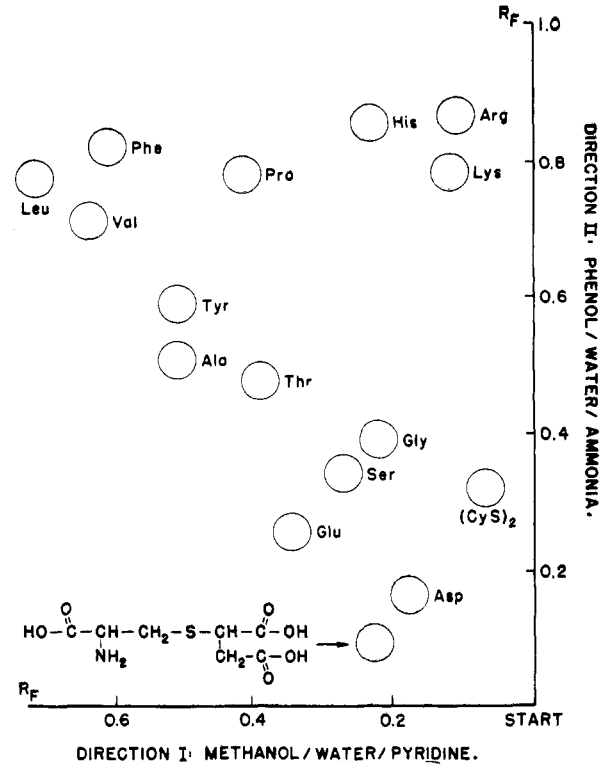

Fig. 1.—Two-dimensional paper chromatography of a hydrolysate of reduced wool treated with MPBM.

pected of wool in which disulfide bonds originally reduced had been partly but substantially replaced by cross-links introduced by the reagents. Thus, the cystine content is decreased by an amount predictable from the reagent uptake if both reactive centers react. The expected cysteine derivative appears in the hydrolysates. The energy needed to extend the treated fibers is less than before reduction, but substantially greater than after reduction and without treatment with the difunctional reagent. Similarly the decrease in supercontraction shows a marked stabilization of the fiber structure. Finally, solubilization of the wool either by hydrolysis or by attack on the disulfide bonds has been greatly restricted.

None of these tests can be interpreted as direct evidence of cross-linking. For example, supercontraction can also be decreased by extensive fiber damage; the 30% index can be increased by other chemical modification. Alkali solubility can be lowered by alkali pretreatment, dissolving part of the more readily attacked wool substance. In our case the necessary tests to eliminate such possibilities are given by parallel experiments with the monofunctional reagent of otherwise similar constitution. In a few instances this gives results similar to, although of lesser magnitude than, those of the difunctional reagents. However, in all instances, except possibly that of the 30% index, the effects of the mono- and difunctional reagents are strongly differentiated. We have the further direct evidence that a bis-maleimide can cross-link the model protein mercaptalbumin. It seems therefore certain that in our instances at least 50% of the disulfide bonds originally present in the wool were

broken by the reducing treatment and that an important fraction of these up to a maximum (in our experiments) equivalent to 42% of the original bonds were replaced by cross-links formed by reaction with the bis-maleimides.

Acknowledgments.—The authors are indebted to I. R. Hunter for advice concerning chromatography, to R. C. Landwehr and Mrs. I. R. Stuart for carrying out some of the physical measurements cited, and to G. E. Secor for the microchemical analyses. They also wish to express gratitude to Dr. H. P. Lundgren for his advice and encouragement during the course of this work.

ALBANY, CAL.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, NEW MEXICO HIGHLANDS UNIVERSITY]

Potential Purine Antagonists. II. Synthesis of Some 7- and 5,7-Substituted Pyrazolo[4,3-d]pyrimidines[1]

BY ROLAND K. ROBINS, FREDERICK W. FURCHT, ALAN D. GRAUER AND JESSE W. JONES

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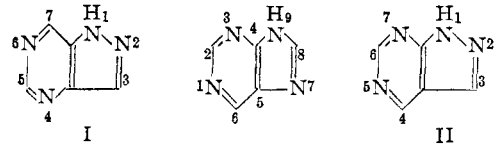
Isomeric structural analogs of various biologically important purines have been synthesized which possess the pyrazolo[4,3-d]pyrimidine ring system. A new route to this ring system has been accomplished beginning with 4-aminopyrazole-3-carboxylic acid. The chemistry of some of the derivatives of pyrazolo[4,3-d]pyrimidine is discussed and a comparison made with the corresponding "isomeric" purine and pyrazolo[3,4-d]pyrimidine ring systems.

In accord with a recent program for synthesis of purine antagonists[2] as potential chemotherapeutic agents against various tumors, it seemed desirable to investigate the synthesis of certain pyrazolo[4,3-d]pyrimidine derivatives. Recent synthesis of the isomeric pyrazolo[3,4-d]pyrimidine[2] ring system has resulted in the preparation of several new compounds with interesting antitumor properties.[3,4]

The structural relationship of pyrazolo[4,3-d]-pyrimidine (I), purine and pyrazolo[3,4-d]pyrimidine (II) is shown below.



I — Pyrazolo[4,3-d]-pyrimidine; Purine; II — Pyrazolo[3,4-d]-pyrimidine

The first reported synthesis of the pyrazolo[3,4-d]pyrimidine ring was that of Behrend[5] who utilized 5-amino-6-methyluracil for the preparation of 5,7-dihydroxypyrazolo[4,3-d]pyrimidine which he called "isoxanthine."

Rose[6,7] has recently accomplished the synthesis of the pyrazolo[4,3-d]pyrimidine ring system (named 1:2:4:6-tetraazaindene by Rose) by diazotization of a 5-amino-6-methylpyrimidine followed by coupling to form the pyrazolo[4,3-d]-pyrimidine ring. This method although quite satisfactory is definitely restricted since a 5-amino-6-methylpyrimidine substituted at position "4" with a hydroxy, mercapto or amino group upon diazotization, couples to give the corresponding oxadiazole, thiadiazole or triazole ring.[7] Because of this difficulty Rose failed to prepare the analog of hypoxanthine, 6-mercaptopurine and adenine in the pyrazolo[4,3-d]pyrimidine series. In view of the work of Rose it seemed advisable to approach the synthesis of these desired derivatives from another route. Success in the synthesis of the corresponding pyrazolo[3,4-d]pyrimidines[2] from a pyrazole intermediate promptly suggested beginning with the pyrazole ring as a route worthy of investigation. The synthesis of 4-aminopyrazole-3-carboxylic (IV) acid by reduction of 4-nitropyrazole-3-carboxylic acid (III) has been reported by Knorr[8] in a footnote, but no experimental details are given. The study of the reduction of 4-nitropyrazole-3-carboxylic acid (III) required large quantities of 3-methyl-4-nitropyrazole from which III was prepared by oxidation with potassium permanganate.[8] The synthesis of 3-methyl-4-nitropyrazole was best accomplished by large scale decarboxylation of 3-methyl-4-nitropyrazole-5-carboxylic acid[9] rather than by nitration of 3-methylpyrazole.[8]

The reduction of 4-nitropyrazole-3-carboxylic acid (III) with sodium hydrosulfite gave 4-aminopyrazole-3-carboxylic acid (IV), in yield superior to any other method of reduction attempted. 4-Aminopyrazole-3-carboxylic acid heated with boiling formamide gave 7-hydroxypyrazolo[4,3-d]-pyrimidine (V), the analog of hypoxanthine, in approximately 30% yield.

4-Nitropyrazole-3-carboxylic acid (III) was esterified to give 4-nitropyrazole-3-ethylcarboxylate (VI) in above 60% yield. Treatment of VI with concentrated ammonium hydroxide gave almost a quantitative yield of 4-nitropyrazole-3-carboxamide (VII). Reduction of 4-nitropyrazole-3-carboxamide (VII) with hydrogen using a palladium-on-charcoal catalyst yielded 4-aminopyrazole-3-carboxamide (VIII). It might be noted that VIII is an isomeric analog of 5-aminoimidazole-4-carboxamide, a known purine precursor in various

(1) Supported in part by a grant-in-aid from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council.

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