[CONTRIBUTION FROM THE DEPARTMENTS OF BACTERIOLOGY AND CHEMISTRY, UNIVERSITY OF KANSAS]

The Interaction of DPN+[1] with *p*-Aminobenzoic Acid and Analogous Compounds

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After DPN+ was caused to react with either PABA,[1] or methyl *p*-aminobenzoate, *p*-dimethylaminobenzoic acid, methyl *p*-dimethylaminobenzoate, *m*-aminobenzoic acid, *o*-aminobenzoic acid and folic acid, it was no longer susceptible to cleavage by DPNase. When the amino group of PABA was acetylated, the resistance to cleavage was greatly reduced. 3,5-Dimethyl-4-aminobenzoic acid produced no inhibition of cleavage. When (DPN·CN)[1] was used, no inhibition was observed. It is suggested that a chemical reaction takes place between DPN+ and PABA forming a new compound upon which DPNase is no longer active. A mechanism for the reaction is postulated, based on the resonance of the pyridinium moiety of the DPN+.

Introduction

In an attempt to extend the work of Woolley, *et al.*,[1-4] on the role of "onium" compounds in metabolism, PABA was selected originally as a possible acceptor amine for the DPN+–DPNase system. It was found that instead PABA inhibited the cleavage of DPN+ by DPNase. In this report data are presented on the nature of the apparent inhibition of DPNase by PABA and related compounds.

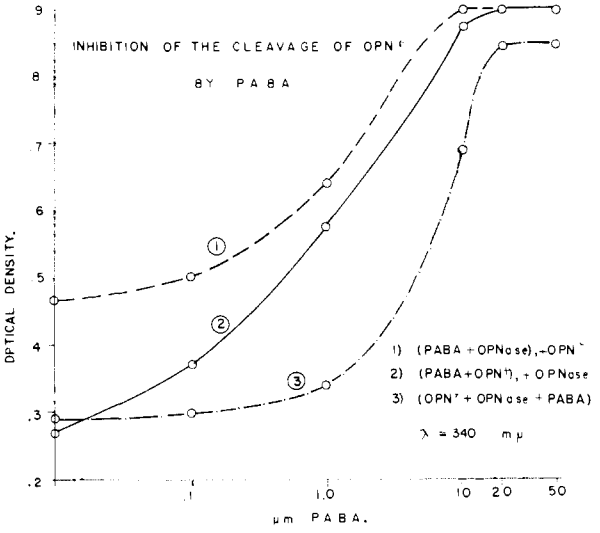

Fig. 1.—Inhibition of the cleavage of DPN+ by PABA: temperature, 37°; incubation time, 24 hr.; *p*H 6.0, phosphate–citrate buffer, Γ/2, 1.2; DPN+, 9 μm./ml. (final concn.); PABA, data expressed as final concn.; final volume, 4 ml.

Materials and Methods

The DPNase used was prepared from beef spleen[5] and purified by ammonium sulfate fractionation. DPNase activity was determined by measuring the change in optical density of the incubated system at 340 mμ after cyanide addition, employing a Beckman DU spectrophotometer.[6]

The incubation of DPN+, DPNase and PABA or related compounds was carried out in a water-bath at 37° with continuous shaking for 24 hours at *p*H 6.0.

Experimental and Results

During the course of studies on the role of "onium" compounds in metabolism, 20 μm. of PABA was treated with 9 μm. of DPN+ in the presence of DPNase. It was observed that PABA apparently inhibited the DPNase activity, as measured by cleavage of DPN+. In an attempt to determine the nature and mechanism of this inhibition, varying amounts of PABA ranging from 0 to 50 μm. were incubated with (a) 1 unit DPNase,[7] (b) 9 μm. DPN+ and (c) 9 μm. DPN+ + 1 unit DPNase. At the end of the incubation period, 9 μm. DPN+ and 1 unit DPNase were added, respectively, to systems (a) and (b) and incubated for another 24 hours. The results obtained are shown in Fig. 1. The optical densities are inversely proportional to the activity of DPNase; that is, the greater the activity of DPNase, the greater the cleavage of DPN+ and hence, the lower the optical density at 340 mμ. It can be seen that the maximum inhibition was obtained at a PABA concentration of 20 μm.; the enzyme is not inactivated: if it were, a linear inhibition should have been obtained in system (a). It thus seemed that PABA and DPN+ may have combined in some manner, thus preventing the enzyme from cleaving the latter.

In an attempt to determine the site of reactivity of the PABA molecule, its functional groups were blocked. *p*-Dimethylaminobenzoic acid, methyl *p*-aminobenzoate and methyl *p*-dimethylaminobenzoate were tested for inhibitory action. The results obtained showed that each one of the compounds tested caused effective inhibition of enzyme action with an optimum inhibition at a concentration of 20 μm. of analog. These observations made it clear that neither the carboxyl nor the amino groups were directly involved in the reaction.

In addition to these data, microbiological assay[8] of the reacted mixtures showed that PABA, as such, did indeed disappear, yet the Bratton–Marshall test[9] for amino groupings indicated an apparent presence of PABA.

Resonance theory was used in an attempt to reconcile the apparent contradictions in the data. The amino group of all the compounds investigated possesses an unshared pair of electrons which can enter into resonance interaction with a benzene ring giving rise to structures in which the *ortho* and *para* carbon atoms bear a formal negative charge and the nitrogen a formal positive charge. Due to resonance the positive charge of the nicotinamide portion of the DPN+ molecule can be shared by the α- and γ-carbon atoms of the ring as well as by the nitrogen atom. With these considerations in mind, the mechanism shown in Fig. 2 is proposed. This mechanism fits all the data which have been obtained in previous experiments: the amino group is free to give the positive Bratton–Marshall test, yet the free PABA has disappeared. A known analogy of this proposed reaction is the condensation of benzoyl chloride, dimethylaniline and

(1) DPN+, diphosphopyridine nucleotide (oxidized); DPNase, diphosphopyridine nucleotidase; PABA, *p*-aminobenzoic acid; (DPN·CN), diphosphopyridine nucleotide–cyanide complex.

(2) D. W. Woolley, *Nature*, **171**, 323 (1953).

(3) (a) D. W. Woolley, THIS JOURNAL, **73**, 1898 (1951); (b) S. G. A. Alivisatos and D. W. Woolley, *ibid.*, **77**, 1065 (1955).

(4) S. G. A. Alivisatos and D. W. Woolley, *J. Biol. Chem.*, **221**, 651 (1956).

(5) L. J. Zatman, N. O. Kaplan and S. P. Colowick, *ibid.*, **200**, 197 (1953).

(6) S. P. Colowick, N. O. Kaplan and M. Ciotti, *ibid.*, **191**, 447 (1951).

(7) 1 unit DPNase is the amount which will cause the splitting of 8 μm. of DPN+ at a temperature of 37° for 24 hours.

(8) S. C. Agarwala and W. H. Peterson, *Arch. Biochem.*, **27**, 304 (1950).

(9) A. C. Bratton and E. K. Marshall, Jr., *J. Biol. Chem.*, **128**, 537 (1939).

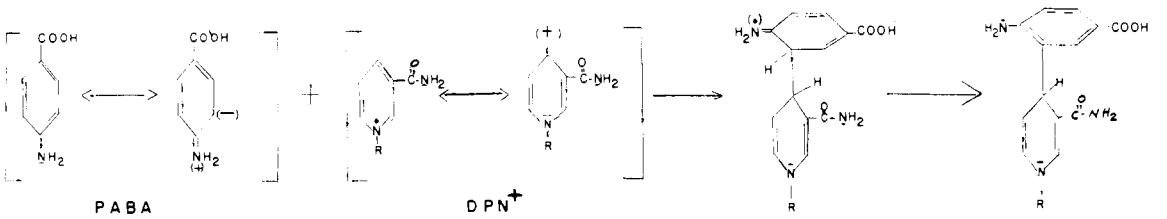

Fig. 2.—Proposed mechanism for the reaction between DPN^+ + PABA.

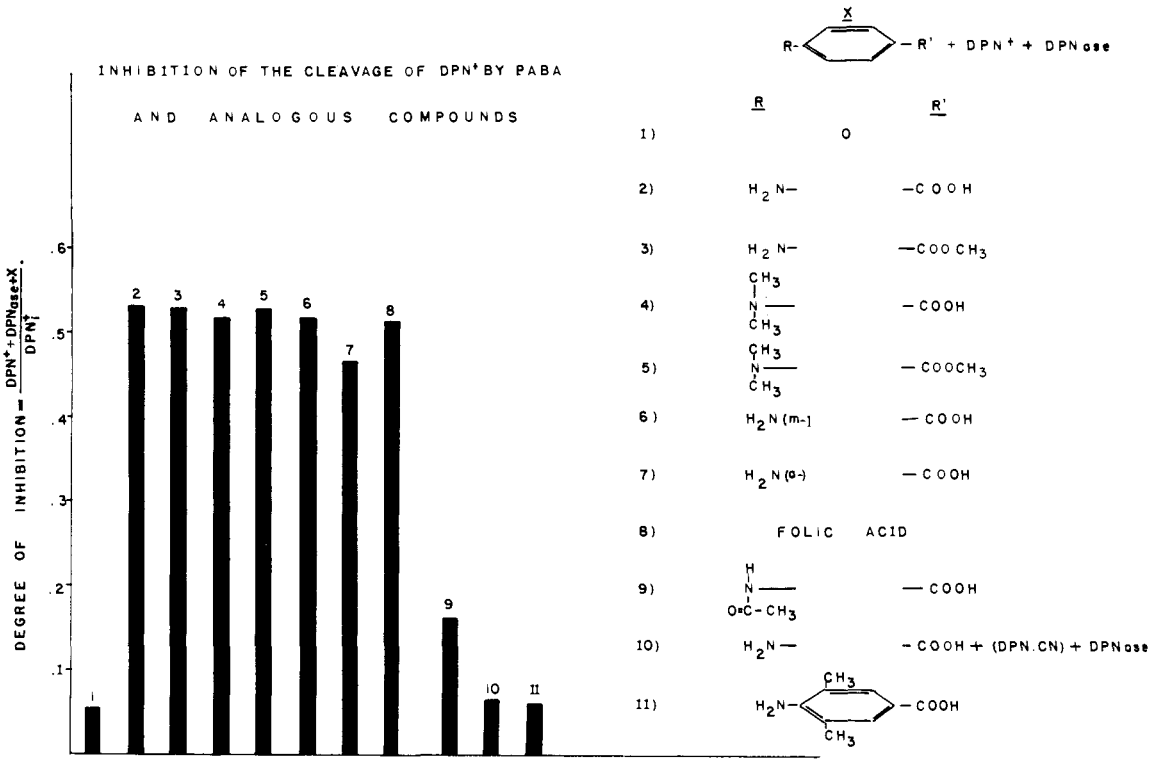

Fig. 3.—Inhibition of the cleavage of DPN^+ by PABA and analogous compounds: temperature, 37°; incubation time, 24 hr.; *p*H 6.0, phosphate-citrate buffer, Γ/2 = 1.2; DPNase, 1 unit/ml. (final concn.); DPN^+, 9 μm./ml. (final concn.); PABA and analogous compounds, 20 μm./ml. (final concn.); final volume, 4 ml.

quinoline to give 1-benzoyl-2-(*p*-dimethylaminophenyl)-1,2-dihydroquinoline.[10]

To further test the proposed mechanism, a series of experiments was carried out. In the first one, the site of reactivity of DPN^+ was blocked by cyanide addition before it was added to the PABA–DPNase system. The results obtained showed no inhibition. Secondly, the amino group of PABA was acetylated. Since the acetyl group is an electron withdrawing group, it competes with the benzene ring for the unshared pair of electrons of the amino group and decreases the reactivity of the compound toward electrophilic substitution. Therefore, this new compound might be expected to show only a fraction of the inhibitory effect shown by PABA. The results obtained showed, as expected, that the apparent inhibition had been reduced to half of that obtained with PABA. In the last experiment, the 3- and 5-positions of PABA were methylated in an attempt to block its sites of reactivity. In this case, no inhibition was obtained which indicated that the reaction between DPN^+ and PABA probably takes place through either the 3- or 5-positions of PABA.

It was also found that *o*- and *m*-aminobenzoic acids and folic acid inhibit the cleavage of DPN^+, as expected, since these compounds would be readily susceptible to attack by an electrophilic agent such as DPN^+ either *ortho* or *para* to the activating amino group. In Fig. 3, all the data obtained from all the investigated compounds are shown. An index of the degree of inhibition of cleavage of DPN^+ is obtained by measuring the ratio of DPN^+ cleavage in the presence of enzyme and the PABA analog. Cleavage of DPN^+ is greatest either in the absence of the analog, when (DPN·CN) is substituted for DPN^+ in the presence of PABA or when 3,5-dimethyl-4-aminobenzoic acid was employed. A lesser degree of apparent inhibition is observed when DPN^+ and DPNase are caused to react in the presence of *p*-acetylaminobenzoic acid. Pronounced inhibition of DPN^+ cleavage is obtained when the (DPN^+ + DPNase) system are brought together in the presence of PABA, *p*-dimethylaminobenzoic acid, methyl *p*-aminobenzoate, methyl *p*-dimethylaminobenzoate, *o*-aminobenzoic acid, *m*-aminobenzoic acid and folic acid.

The absorption spectra of the following systems were studied: DPN^+, DPN^+ + acetyl *p*-aminobenzoic acid, DPN^+ + 3,5-dimethyl-4-aminobenzoic acid, DPN^+ + methyl *p*-aminobenzoate, DPN^+ + *p*-dimethylaminobenzoic acid and DPN^+ + methyl *p*-dimethylaminobenzoate. All of these systems were incubated at 37° for 24 hours at *p*H of 6.0. Controls, consisting of the analogs described only, were incubated simultaneously with the experimental vessels under precisely the same conditions. This was done to test the possibility of a new compound arising due to the oxidation of the aromatic amine instead of to the formation of the proposed compound. The results obtained are shown in Fig. 4. In part (a) where the controls are examined, it can be seen that when PABA and related compounds are incubated in the absence of DPN^+ no absorption peak at 310 mμ is obtained. However, part (b) of Fig. 4 shows that the

(10) W. E. McEwen, R. H. Terss and I. W. Elliott, THIS JOURNAL, **74**, 3605 (1952).

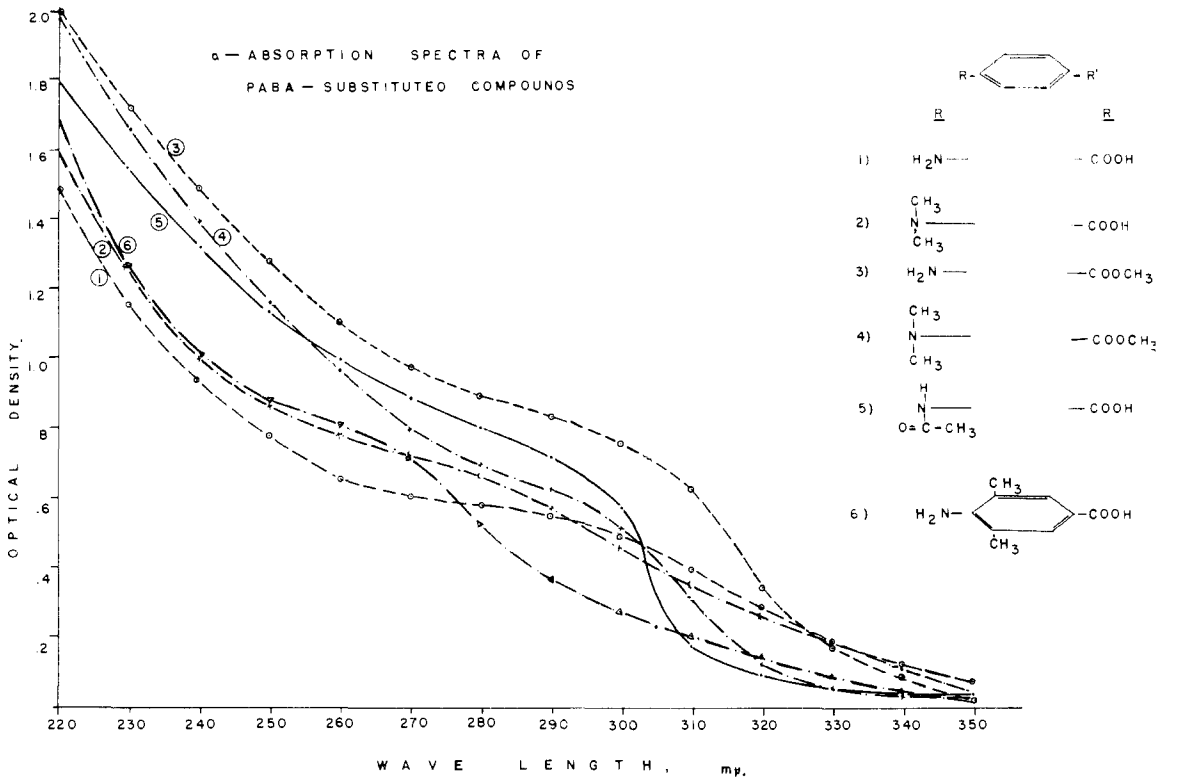

Fig. 4A.—Absorption spectra of PABA and PABA-substituted compounds: temperature, 37°; incubation time, 24 hr.; *p*H 6.0, phosphate–citrate buffer, Γ/2 = 1.2; PABA and PABA substituted compounds, 20 μm./ml (final concn.); final volume, 3 ml.

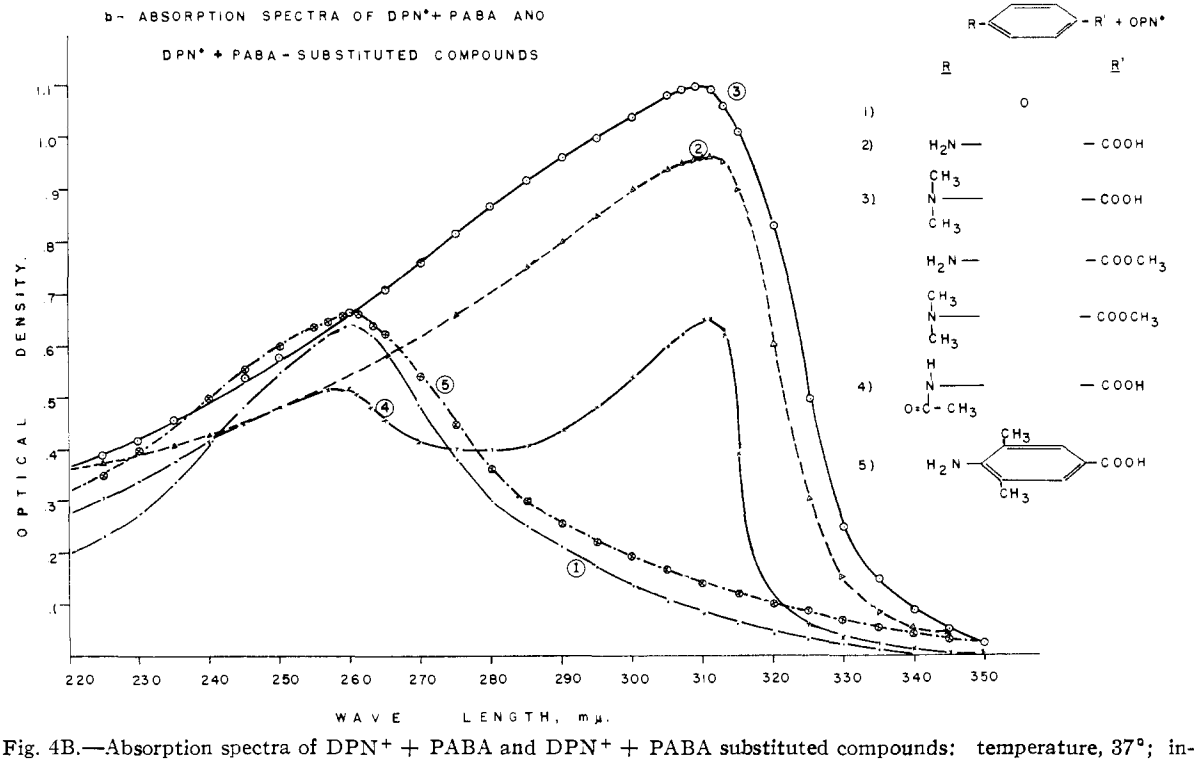

Fig. 4B.—Absorption spectra of DPN^+ + PABA and DPN^+ + PABA substituted compounds: temperature, 37°; incubation time, 24 hr.; *p*H 6.0, phosphate–citrate buffer, Γ/2 = 1.2; DPN^+, 9 μm./ml. (final concn.); PABA and PABA substituted compounds, 20 μm./ml. (final concn.); final volume, 3 ml.

reaction product of DPN^+ and PABA or related compounds in which the sites of reactivity have not been blocked do give a peak at 310 mμ, indicating the presence of a new compound.

The reaction product of DPN^+ + PABA and DPN^+ + PABA-substituted compounds were isolated by paper chromatography (1:1 mixture of ethanol and 0.1 *N* acetic acid, ascending, 20 hours, Whatman #1 paper). The spots on the chromatogram were detected with a "Blak-Ray," long wave ultraviolet source. The following R_f values were obtained: DPN^+ = 0.62, DPN^+ + PABA = 0.84, DPN^+ + methyl *p*-aminobenzoate = 0.83, DPN^+ *p*-dimethylaminobenzoic acid = 0.83, DPN^+ + methyl *p*-dimethylaminobenzoate = 0.84, DPN^+ + *p*-acetylaminobenzoic acid = 0.62, DPN^+ + 3,5-dimethyl-4-aminobenzoic acid = 0.62. The spots were eluted from the paper with distilled water and absorption spectra of the eluted material were determined. DPN^+ and DPN^+ + 3,5-dimethyl-4-PABA showed an absorption peak at 260 mμ; DPN^+ + *p*-acetylaminobenzoic acid showed two absorption peaks, one at 260 mμ (R_f 0.62) and the other at 310 mμ (R_f 0.84). DPN^+ + PABA or methyl *p*-aminobenzoate, *p*-dimethylaminobenzoic acid and methyl *p*-dimethylaminobenzoate showed absorption peaks at 310 mμ.

Discussion

Kaplan[11] has reviewed many DPN^+ addition reactions. In this report, on the basis of the data presented, a new type of reaction is presented for consideration. PABA has been found to inhibit the cleavage of DPN^+ by DPNase. The inhibition probably comes about when DPN^+ combines chemically with PABA forming a new compound for which DPNase is no longer specific. Neither the carboxyl nor the amino groups are directly involved in the reaction. The site of reactivity of PABA is likely the 3- or 5-position. Blocking these positions by methylation is shown to prevent inhibition of DPN^+ cleavage probably because the dihydrobenzene ring of the adduct presumably formed cannot regain its aromaticity by loss of proton as in the case of PABA reaction. Acetylation of the amino group decreases the inhibition as expected, since the acetyl group competes with the ring for the pair of unshared electrons of the amino group and decreases the reactivity of the aromatic ring toward electrophilic substitution. *o*-Aminobenzoic acid and *m*-aminobenzoic acid were also found to inhibit DPN^+ cleavage. This is expected since there are positions *ortho* and *para* to the amino group at which an electrophilic substitution reaction involving attack by DPN^+ can occur.

The evidence of DPN^+ reactivity with PABA and related compounds raises the question of the situation existing in the intact cell. Not only does PABA inhibit DPN^+ cleavage, but folic acid, the naturally occurring combined form of PABA, is likewise inhibitory. We are investigating the interrelationships of the cellular co-factors on the basis of chemical structures, in light of the present data and hypothesis.

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[CONTRIBUTION FROM THE FRICK CHEMICAL LABORATORY, PRINCETON UNIVERSITY]

Pteridines. XVI. A Synthesis of 2-Aminopyrazine-3-carboxamides by Reductive Ring Cleavage of 3-Hydroxy-1-pyrazolo[b]pyrazines[1-3]

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A new method for the synthesis of 2-amino- and 2-substituted aminopyrazine-3-carboxamides has been developed which involves the following steps: (1) the synthesis of suitably substituted 3-hydroxy-4,5-diaminopyrazoles, (2) ring closure of these intermediates by reaction with α,β-dicarbonyl compounds to give 3-hydroxy-1-pyrazolo[b]pyrazines, and (3) reductive ring cleavage by means of Raney nickel of the –N–N– bond of the 3-hydroxy-1-pyrazolo[b]pyrazines. Each of these steps is considered in detail. Since previous work has shown that pteridines may be formed by ring closure of 2-aminopyrazine-3-carboxamides, the above reaction sequence constitutes a new total synthetic approach to pteridines.

The construction of the bicyclic pteridine ring system may be approached from either of two directions. In the first, a suitable pyrimidine intermediate is initially prepared and the pyrazine ring is closed in the terminal stage of the synthesis. The most widely employed method for the preparation of pteridines, which involves the condensation of a 4,5-diaminopyrimidine with an α,β-dicarbonyl compound, is of this type.[5] Although several variations of this approach have been devised,[6-11] the over-all method suffers from inescapable limitations which previously have been pointed out.[12] In the second approach, a pyrazine intermediate is prepared initially and the fused pyrimidine ring is closed in the terminal stage of the synthesis.[12-20] This method has not been widely

(1) A preliminary note describing the results of the present investigation has been published (T. S. Osdene and E. C. Taylor, THIS JOURNAL, **78**, 5451 (1956)).

(2) This work was supported by a grant from the American Cancer Society.

(3) Presented before the Division of Organic Chemistry at the 131st National ACS Meeting in Miami, Fla., April 7–12, 1957.

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