Journal of Medicinal Chemistry, 1974, Vol. 17, No. 2 223

Synthesis and Biological Evaluation of Isofolic Acid†

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The synthesis of isofolic acid has been accomplished by utilizing an unambiguous procedure. A new ring closure reaction has been developed for the construction of the isofolate framework and substituted pteridines in general. Isofolic acid and isopteroic acid were tested for their ability to inhibit the growth of two folate-requiring strains of bacteria. Unlike homofolic acid, which is active only in the reduced form, isofolic acid derives its merit as one of the first folate analogs possessing a normal 2-amino-4-hydroxypteridine moiety showing remarkable antimetabolic activity *per se*.

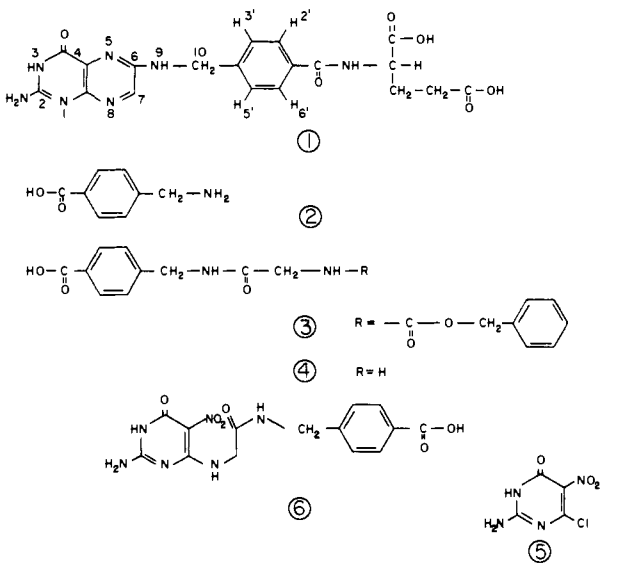
During the approximately 25 years since methotrexate† and aminopterin† became available, a very large number of analogs of the vitamin folic acid† have been prepared.[1,2] The great majority of these compounds possess the 2,4-diaminopyrimidine structure and are directed at the enzyme dihydrofolate reductase (DHFR).† The very strong binding of this type of analog to DHFR, which has been described as stoichiometric[3] and noncompetitive,[4] is directly related to the clinical toxicity which seriously hampers their use in chemotherapy. Although the high degree of specificity of this type of analog appears desirable, one can envision interference with one-carbon metabolism in situations where a growing tissue is making demands on thymidylate synthetase with the concomitant accumulation of dihydrofolic acid. Thus, a deficit of folate coenzymes in general could result from the inhibition of DHFR. These thoughts have led to a consideration of new types of analogs for use in parasitic and cancer chemotherapy. These compounds would possess a normal 2-amino-4-hydroxy-6-substituted pteridine which would not be expected to inhibit DHFR in a noncompetitive manner and, indeed, may serve as substrate for this enzyme. They would instead be altered in the region corresponding to the C^9–N^{10} bridge in folic acid. Studies with homofolic acid,† which is reduced by DHFR, have given impetus to this work.[5-7]

In recent years a number of variations of the original synthesis of the vitamin folic acid[8] have appeared in the literature. These include the synthesis of folate antagonists such as methotrexate and aminopterin.[9] These reactions involve the condensation of an appropriately substituted 5,6-diaminopyrimidine with a 1,2-dicarbonyl compound to give rise to two isomeric products.

Careful theoretical considerations precluded the applicability of any of the above modifications to the synthesis of isofolic acid, due to the requirement that a substituted amino group must be attached to C_6 of the pteridine moiety. The synthesis of 2,6-diamino-4-hydroxypteridine is described by Stuart, West, and Wood[10] by a modification of the Boon and Leigh method,[11] but no *N*-alkyl derivatives have been prepared. A previous attempt[12] to prepare 2,6-diamino-7,8-dihydro-4-hydroxypteridine from 2-amino-4-carbamoylmethylamino-6-hydroxy-5-nitropyrimidine by Wood and coworkers resulted in the formation of xanthopterin.

We here describe the novel and unambiguous synthesis of a 2,6-diamino-4-hydroxypteridine which is substituted at the 6-amino function as exemplified in the synthesis of isofolic acid (**1**), Schemes I and II.

Scheme I



Conversion of commercially available *p*-carboxybenzaldehyde to α-amino-*p*-toluic acid (**2**) was accomplished recently.[13] The amino function of **2** was then coupled with the carboxyl group of *N*-carbobenzyloxyglycine in an amide linkage by the isobutyl chloroformate method[14,15] to **3**. Hydrogenation of **3** resulted in the removal of the carbobenzyloxy group to give dipeptide **4**. Nitration of 2-amino-6-chloro-4-hydroxypyrimidine in a manner reported earlier gave the corresponding 5-nitro compound **5**.[16] The free amino group of **4** was then attached to C_6 of the pyrimidine by a displacement reaction. The crucial intermediate **6** was thus obtained in reasonably good yield. Reduction of the nitro group of **6** proceeded smoothly with sodium dithionite at pH 7.0 to the corresponding amino compound **7**, which was found to be very unstable in air. Great care had to be taken in the handling of this compound.

Several attempts were made to effect the dehydrocyclization of **7** to 7,8-dihydroisopteroic acid (**12**). The use of recommended dehydrating agents[17-20] resulted either in loss of the side chain or tarry products with the formation of 7,8-dihydroxanthopterin (**11**) and **2** as noted by Wood and coworkers.[10] The structures of **11** and **2** were confirmed by their spectral characteristics and comparison with authentic samples after oxidation of the dihydro intermediates with MnO_2 in NaOH solution.

The mechanism by which the observed product might result from the attempted ring closure reaction is shown in Scheme I. The acid-catalyzed attack of the 5-amino group on the carbonyl function resulted in the formation of **8**. Protonation of N^9 or the hydroxyl at C_6 then facili-

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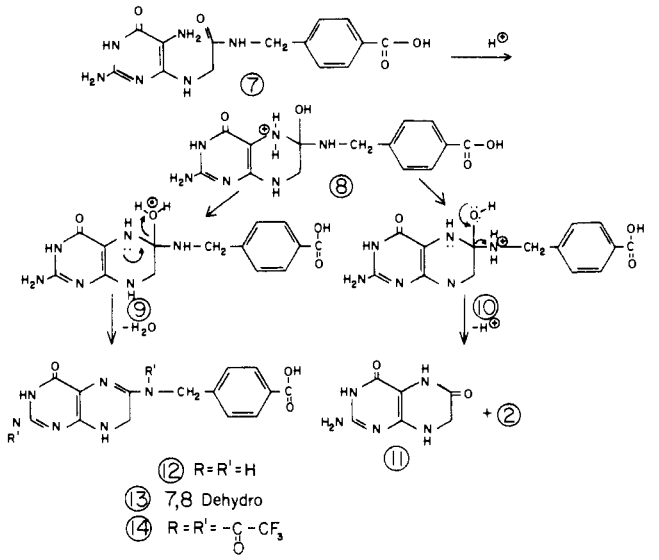
†Trivial names in general usage will be used for these compounds: isofolic acid = *N*-[*p*-[[(2-amino-4-hydroxy-6-pteridinyl)amino]methyl]benzoyl]-glutamic acid; methotrexate = *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]-methylamino]benzoyl]glutamic acid; aminopterin = *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]amino]benzoyl]glutamic acid; folic acid = *N*-[*p*-[[(2-amino-4-hydroxy-6-pteridinyl)methyl]amino]benzoyl]glutamic acid; homofolic acid = *N*-[*p*-[[(2-amino-4-hydroxy-6-pteridinyl)ethyl]amino]benzoyl]-glutamic acid. Other abbreviations include: DHFR, dihydrofolate reductase; TFAn, trifluoroacetic anhydride; DMSO, dimethyl sulfoxide; THF, tetrahydrofuran; DEAE, diethylaminoethyl; DMF, dimethylformamide.

Scheme II



tated the formation of intermediates **9** and **10**. The loss of the side chain from intermediate **10** by a concerted process is straightforward. The consistent and exclusive formation of xanthopterin when acid catalysts[18] were employed prompted us to investigate in detail the conditions under which the desired **12** might be formed. The syntheses of several 8-arylpurines have been carried out from 2-amino-5-arylamidopyrimidines by the use of several reagents and also by dry heating.[17-20] Although such an approach was unprecedented in the construction of pteridine ring systems, the failures encountered were made to order for the pyrolysis of **7** without the use of an acid catalyst. Therefore, **7** was heated neat to its melting point of 245–250° and the product oxidized in base with MnO_2. The desired compound **13** was isolated in about 3% yield from **7**.

Several modifications of this simple pyrolytic reaction were considered as a means to improve the yield of **12**. One approach was the pyrolysis of **7** in a suitable solvent system to which a dehydrating agent, such as molecular sieve, could be added. To minimize the intramolecular proton transfer from N^5 to N^9, the introduction of a strong base, diazabicyclooctane, in the reaction was also considered. If the added base removed a proton from N^5 of intermediate **8a**,‡ **12** might be formed after reprotonation of the oxygen at C_6 and dehydration. These speculations were substantiated when it was observed that by refluxing **7** with 2-ethoxyethyl ether in the presence of excess diazabicyclooctane and molecular sieve in a nitrogen atmosphere for 2 hr, and subsequently oxidizing the product with MnO_2 in NaOH and purifying by ion exchange chromatography, a yield of 10–15% of **13** was obtained. However, xanthopterin was still formed in an appreciable amount.

The structure of **13** was established beyond doubt by physical and chemical methods. The remaining problem was the addition of a glutamyl residue to the pteroic acid analog **13**. Treatment of **13** with trifluoroacetic anhydride (TFAn)† at room temperature for several days resulted in the formation of a trifluoroacetyl derivative. The product was then dissolved in a 1:1 mixture of DMSO† and TFH† and the carboxyl group of the pteroic acid analog **14** activated by the isobutyl chloroformate method.[14] The mixed anhydride of **13** and isobutyl chloroformate was then coupled to a resin ester of glutamic acid. The glutamyl resin ester was prepared by esterification (reflux 48 hr in absolute EtOH) of *tert*-butyloxycarbonylglutamate α-benzyl ester to the Merrifield resin.[15] After removal of the *tert*-Boc protection group the glutamyl resin ester was allowed to react for 2 hr with the mixed anhydride of **13**. Isofolic acid (**1**) was cleaved from the resin and simultaneously deprotected as described below.

‡The intermediate **8a** is the result of the pyrolytic reaction without any added catalyst and therefore bears a negative charge on the oxygen at C_6. Transfer of a proton from N^5 is then possible to either the oxygen at C_6 or the N^9 nitrogen atom.

Table I. Amount of Isofolic and Isopteroic Acid (g/tube) Required for 50% Inhibition of Growth of Two Folate-Requiring Bacteria

Compound	*S. faecium*[a] ATCC No. 8043	*L. casei*[b] ATCC No. 7469
Isopteroic	2.5×10^{-9}	No inhibition at 1×10^{-4}
Isofolic	2.5×10^{-9}	9×10^{-8}

[a] 2.5×10^{-9} g of folic acid present in each assay tube. [b] 5.0×10^{-10} g of folic acid present in each assay tube.

The crude isofolic acid thus obtained was purified by chromatography. The nmr spectrum of this compound in 0.1 *N* NaOD in D_2O with sodium 3-trimethylsilylpropionate-*2,2,3,3-d*$_4$ (TSP) as internal standard showed the resonance of the C_7 proton as a singlet at 8.10 ppm which is 28 Hz more shielded compared to the C_7 resonance of folic acid under identical conditions, and the $H_{3',5'}$ resonances were deshielded 42 Hz, resulting from the interchange of the methylene and amino groups, observations in complete harmony with the proposed structure.

Discussion

Although analogs of folic acid play an important role in cancer chemotherapy, relatively few structural modifications have been carried out on this molecule. It is unfortunately a measure of the present state of antifolate art that it is not possible to stipulate the structure-activity relationships with reasonable accuracy. The mere fact that a reversal of the substituents at the 9–10 bridge region could elicit antimetabolic activity is in direct contradiction to the widely accepted notion that an ideal antifol must possess a 2,4-diaminopyrimidine moiety.[2]

The synthesis of isofolic acid thus not only contributes to a broader understanding of structure-activity relationships but also to the generation of a new class of antifols with the isofolate framework.

The potencies of isopteroic acid and isofolic acid as inhibitors of *S. faecium* and *Lactobacillus casei* are close to that of methotrexate. It is interesting to note that, as shown in Table I for *S. faecium*, there appears to be no irreversible binding of these analogs to any enzyme. The use of equivalent amounts of isofolic or isopteroic and folic acid over a wide range of concentrations invariably results in 50% inhibition of growth over the tubes containing folic acid alone.

The fact that isopteroic acid does not inhibit *L. casei* even at 5 orders of magnitude higher concentration than required to inhibit *S. faecium* is significant. It should be noted that *L. casei* cannot utilize pteroic acid in place of folic acid, presumably due to its inability to attach the glutamyl residue. These results give great encouragement to the idea that these analogs are functioning as folate analogs and must therefore possess the glutamyl moiety. These considerations suggest that isopteroic acid may have merit as an antibacterial agent with a specificity similar to that of the sulfonamides. Those organisms capable of attaching the terminal glutamyl residue would be inhibited while those incapable of performing this biosynthetic reaction, such as man, all other mammals studied, and bacteria such as *L. casei*, would not.

Table II. Reversal of Isofolic Acid Inhibition of *Streptococcus faecium* ATCC No. 8043 Growth by Folic Acid and Thymidine

Isofolate/tube	Folate/tube	Methionine	Adenine	Thymidine	Turbidity
	5×10^{-9} g				0.85
5×10^{-9} g					0.00
5×10^{-9} g		To 1×10^{-3} g			0.00
5×10^{-9} g			To 1×10^{-3} g		0.00
5×10^{-9} g				2×10^{-5} g	0.85
5×10^{-9} g	5×10^{-9} g				0.43

The reversal studies in Table II suggest that isofolic acid is functioning preferentially to inhibit the synthesis of thymidylic acid, since other folate-related metabolites were without effect. These results must now be confirmed on isolated thymidylate synthetase.

Preliminary experiments conducted in our laboratory substantiate that the unique ring closure of **7** to **12** is quite general and does not depend on substituents at C_4.

It had been reported by Viscontini and Piraux[21] that 2,6-diamino-7,8-dihydro-4-hydroxypteridine is very unstable and is converted to xanthopterin just by crystallization from water. Since xanthopterin was a major side product in our synthesis, it occurred to us that indeed the ring closure of **7** to **12** might have gone well and that the resulting dihydro derivative is unstable and xanthopterin arises during the oxidation step by the addition of water across the polar 5,6 C=N bond,[22-25] and subsequent ejection of the side chain. However, our results with regard to this phenomenon do not confirm the observation made by Viscontini and Piraux. Reduction of isopteroic acid to the 7,8-dihydro derivative was carried out with sodium dithionate as described earlier for several folate analogs[26,27] and pteridines.[12] After destruction of excess dithionite the mixture was oxidized back at pH 13 with MnO_2 and the product examined by chromatography. No trace of xanthopterin was observed in the reaction mixture. Therefore, dihydroxanthopterin was formed during the ring closure step as outlined in Scheme I. Further evidence for the proposed mechanism in Scheme I was obtained when **7** was pyrolyzed in a hydrocarbon solvent at 260°, and the volatile materials were swept away from the reaction vessel with a stream of nitrogen to the surface of a cold finger kept at −70°. A white crystalline material was deposited on the cold finger. This was subsequently identified as the side chain, *viz.*, **2**. The interesting biological properties exhibited by this molecule warrant further exploration of reagents and conditions to carry out the ring closure reactions more effectively.

Experimental Section

Melting points are uncorrected and were determined on a Fisher-Johns apparatus. Nmr spectra were run in 0.1 *N* NaOD in D_2O on a 60 MHz, HA-60 Varian spectrometer or Varian X1-100 with TMS as lock signal. Field strengths of the various proton resonances are expressed in parts per million and coupling constants as cycles per second. Peak multiplicity is depicted as usual: s for singlet, d for doublet, t for triplet, q for quartet, and c for complex. Ultraviolet spectra were determined on a Beckman DU or a Bausch and Lomb Spectronic 505 spectrophotometer. All chromatography was carried out on DEAE cellulose (Mannex DEAE, from Schwarz/Mann, Orangeburg, N. Y.) in the chloride form with 1.2 × 22 cm packing. A linear NaCl gradient, 0.005 *M* phosphate buffer, pH 7.0, from zero to 0.5 *M* with respect to NaCl, was used to elute the column in a total volume of 2 l. Infrared spectra were run on a Beckman infrared spectrophotometer. Elemental analyses were by Galbraith Laboratories, Inc., Knoxville, Tenn.§ Yields represent the actual amount of pure compound isolated, assuming 100% reaction.

§Where analyses are indicated only by symbols of the elements, analytical results obtained for these elements or functions were within ±0.4% of the theoretical values.

Preparation of 3. Carbobenzyloxyglycine (1 mmol) was dissolved in 9 ml of THF and 1 mmol of *N*-methylmorpholine added. The mixture was kept for 15 min in an ice bath. Then 1 mmol of isobutyl chloroformate was added with stirring; after 15 min 1 mmol of **2** was added in 70 ml of cold THF. The reaction was allowed to proceed at room temperature for 18 hr after which time the solvent was taken off under vacuum and the residue triturated with 50 ml of H_2O, then filtered, and recrystallized from EtOH: yield 65%; mp 191-192°. *Anal.* ($C_{18}H_{18}N_2O_5$) C, H, N.

Hydrogenation of 3. The catalytic hydrogenation of **3** was carried out in 95% EtOH with 5% Pd/C at 40 psi for 18 hr. Removal of catalyst by filtration and washing with hot AcOH gave a solution which on evaporation furnished compound **4** in quantitative yield: mp 272°. *Anal.* $C_{10}H_{12}N_2O_3$) C, H, N.

Preparation of 6. **4** (1 mmol) was dissolved in 20 ml of water at 100° in a round-bottomed flask with stirring. **5** (1 mmol) was dissolved in 50 ml of 95% EtOH and the hot solution slowly added to the boiling aqueous solution of **4** through a dropping funnel. After the addition, reflux was continued for 3 hr, after which time crystals were formed. After cooling, **6** was isolated by filtration and recrystallized from a DMSO-benzene mixture in 70% yield: mp >300°. *Anal.* ($C_{14}H_{14}N_6O_6$) C, H, N.

Reduction of 6 with Dithionite. **6** (100 mg) was suspended in 10 ml of water and 1 *N* NaOH added to the solution so that the pH did not exceed 10. When all the material was in solution the pH was lowered to 7.2 and it was heated to 50°. Solid dithionite (300 mg) was added portionwise; the solution became colorless. The solution was chilled and a white solid was collected by filtration and washed three times with 10 ml of ice water and finally with absolute EtOH. This was immediately dried under vacuum at room temperature and found to be stable for 24 hr: yield 70 mg.

Pyrolysis of 7 and Preparation of Isopteroic Acid (13). Of the numerous variations of this reaction, this is typical of the one which gave the best result. In a three-necked dry round-bottomed flask was placed 10 g of Linde molecular sieve type 3A and 35 ml of dry 2-ethoxyethyl ether. Nitrogen was bubbled through the solution for 5 min and 2 drops of mercaptoethanol added with stirring. Then a mixture of 600 mg of **7** and 1 g of diazabicyclooctane was added and the flask quickly heated to reflux under nitrogen. The reflux was continued for 2 hr and cooled. Then 100 ml of diethyl ether was added to the reaction mixture and filtered. The precipitate with the molecular sieve on the funnel was then washed several times with small portions of 0.1 *N* NaOH solution so that the total volume did not exceed 150 ml. The washings were combined, and 5 g of solid MnO_2 was added to the solution and allowed to stir for 8 hr at room temperature in an open beaker. After this period the MnO_2 was removed by filtration. The green fluorescent solution thus obtained was adjusted to pH 7.3 by addition of 1 *N* HCl, applied on a 3 × 30 cm column of DEAE Cl^-, and eluted with 1 l. of 0.06 *M* NaCl solution. The bright yellow band which was eluted at this concentration was identified as xanthopterin by comparison with an authentic sample.

The column was then eluted with 0.125 *M* NaCl and isopteroic acid was obtained as a bright fluorescent compound in the final liter of the total of 1.5 l. used to elute the column. The solution was acidified with 5 ml of glacial AcOH and kept at 0° for 18 hr. The precipitate thus formed was collected by filtration, washed several times with water, and dried under vacuum at 60° over P_2O_5: yield 90 mg; λ_{max} (in 0.1 *N* NaOH) 406 (6292), 265 (21,520), and 241 nm (21,330). *Anal.* ($C_{14}H_{12}N_6O_3$) C, H, O; N: calcd, 26.17; found, 25.25.

Preparation of Trifluoroacetylisopteroic Acid and Isofolic Acid (1). Isopteroic acid (100 mg) was stirred with 15 ml of TFAn for several days at room temperature in a round-bottomed flask until all the material was in solution. The TFAn was removed *in vacuo* at 40° and 20 ml of dry benzene added. The benzene was removed under vacuum, and the process repeated twice. Ice (10 g)

was added and the gum was triturated with a spatula. The mixture was then freeze-dried and again dried at 60° under vacuum over P_2O_5 for 24 hr.

The trifluoroacetyl derivative thus obtained was dissolved in 10 ml of DMF and 1.25 equiv of *N*-methylmorpholine added with stirring at 0°. Isobutyl chloroformate (1 equiv) was added and the solution maintained at 0° for 15 min. Glutamic acid diethyl ester (2 equiv) in 10 ml of DMF was next added to the mixed anhydride at 0° and kept at that temperature for 1 hr. The solvent was removed *in vacuo* at 60° and the residue suspended in 0.5 *N* NaOH, heated to 90° in a nitrogen atmosphere, and held for 45 min, when a clear solution was obtained. After cooling, the pH was lowered to 2 and the precipitate collected by centrifugation. This was dissolved in 20 ml of 0.1 *N* NaOH and the pH lowered to 7.3, diluted to 250 ml, applied on a standard DEAE Cl^- column, and eluted with a linear NaCl gradient.

Isofolic acid was eluted at 0.19 *M* NaCl as a single band. The eluates corresponding to this peak were combined, evaporated to 15 ml, and cooled, and the pH was lowered to 2. The golden yellow isofolic acid thus obtained was washed several times with H_2O and dried. The yield was 35%. This undesirable yield in the standard peptide coupling procedure employed prompted us to examine this reaction by a slightly modified solid-phase peptide synthetic procedure. *tert*-Butyloxycarbonyl-L-glutamic acid α-benzyl ester was attached to the Merrifield chloromethyl peptide resin through the γ-carboxyl end. The mixed anhydride was made in a 1:1 mixture of DMSO–THF, added to the esterified resin, and the coupling reaction allowed to proceed overnight. The product was cleaved from the resin by use of a 1:1 mixture of *p*-dioxane–2 *N* NaOH for 1 hr at room temperature, then 20 min at 50°. The final purifications were as described above: yield 75–80% λ_{max} (at pH 7) 400, 284, and 235 nm; λ_{max} (in 0.1 *N* NaOH) 408 (4722), 265 (21,520), and 241 nm (21,330); λ_{max} (in 0.1 *N* HCl) 385, 280, and 265 nm; nmr signals (in 0.1 *N* NaOD in D_2O) 8.1 (s, one proton, H_7), 7.8 (d, J = 8 Hz, two protons, $H_{2',6'}$), 7.48 (d, J = 8 Hz, two protons, $H_{3',5'}$), 4.62 (t, J = 6 Hz, α proton of glutamic acid), and between 2 and 2.5 ppm (c, four protons, glutamic acid). *Anal.* ($C_{19}H_{19}N_7O_6$) C, H, N, O.

Biological Evaluation. Isofolic acid and isopteroic acid were tested for their ability to inhibit the growth of two folic acid requiring strains of bacteria, *Streptococcus faecium* ATCC No. 8043 and *Lactobacillus casei* ATCC No. 7469. These studies were carried out in duplicate tubes containing the appropriate Difco folic acid assay medium for these organisms. The results of these inhibition studies are summarized in Table I.

A number of folic acid related metabolites were used in attempts to reverse the inhibition brought about with isofolic acid in *S. faecium*. Table II shows that only thymidine and folic acid were able to reverse the inhibition.

Acknowledgments. This work was supported by Grant No. 1C-3M of the American Cancer Society, Inc., and is Contribution No. 1218 of the U. S. Army Research Program on Malaria, Grant No. DADA17-73-G-9382. The technical assistance of Mrs. Barbara Hudson and Miss Eleanor Braverman is gratefully acknowledged.

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