type containing 35 chromosomes. Interbreeding between heterozygotes would produce animals with 2n = 34 and karyotypes roughly similar to those observed in *nevadensis* and *elegans*. Following centric fusion a pericentric inversion must have occurred to alter the metacentric produced by fusion of equal sized acrocentrics to a submetacentric. Finally, remodeling of the Y chromosomes must be postulated because the Y of *nevadensis* and *elegans* is larger than that of *richardsonii*. When peripheral populations containing the polymorphism became isolated from the parental stock, animals with 2n = 35 and 2n = 36 were selected against and eliminated, resulting in a population containing only the new 2n = 34 (*nevadensis* and *elegans*) karyotypes. Because centric fusion is a more likely mechanism than centric fission or dissociation for conversion of the *richardsonii* karyotype to *elegans, richardsonii* is considered chromosomally more ancestral(3).

It is not possible to determine whether *nevadensis* and *elegans* evolved from one or two isolates that became separated from the ancestral *richardsonii* population. Possibly the earlier distribution of the 2n = 34 population was much larger than it is at present and as climatic conditions became unfavorable *nevadensis* remained as a relict population in Nevada and diverged from *elegans*, at the same time retaining a similar karyotype. In any case chromosomal characters suggest that *nevadensis* and *elegans* are more closely related to one another than either is to *richardsonii*.

Summary. Chromosomes were analyzed from the bone marrow of 4 specimens of *Spermophilus richardsonii nevadensis*. Comparison of chromosomal characters from the 3 geographically isolated subspecies of *S. richardsonii* demonstrates that *S. r. nevadensis* and *S. r. elegans* both have a diploid number of 34 and similar karyotypes whereas *S. r. richardsonii* has a diploid number of 36 and a different karyotype. The *nevadensis* and *elegans* karyotypes probably evolved from the chromosomally more ancestral *richardsonii* karyotype by means of a chromosomal polymorphism. The data also suggest that *S. r. nevadensis* and *S. r. elegans* are more closely related to one another than either is to *S. r. richardsonii*.

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Antiparasitic Drugs. V. Anticoccidial Activity of 4-Amino-2-Ethoxybenzoic Acid and Related Compounds. (29616)

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Wyss(1), Johnson(2), Martin(3) and others have tested ring-substituted 4-aminobenzoic acids as inhibitors of the growth of 4-aminobenzoic acid (PABA)-requiring bacteria. 4-Amino-2-chlorobenzoic acid has received the widest attention, *in vitro* activity being noted with *E. coli, D. pneumoniae, S. hemolyticus, A. aerogenes* and the tuberculosis organism. *In vivo* trials, however, were disappointing(1a).

The sensitivity of coccidia to PABA antagonists of the sulfanilamide and 4,4′-di-

aminodiphenylsulfone types has long been recognized; in fact, sulfaquinoxaline was the first practical agent for control of coccidiosis in poultry(4,5). Combinations of diaminopyrimidines, such as pyrimethamine, or diaminodihydrotriazines with sulfa drugs are also effective(6). In such combinations potentiation is observed, probably due to sequential blocks of folic acid synthesis and reduction.

These considerations suggested evaluation of some ring-substituted PABA compounds in coccidiosis. It was found that 4-amino-2-chlorobenzoic acid (II), while inactive against *Eimeria tenella* at 0.1%, controls laboratory *E. maxima* infections at a feed level of 0.0125%. 4-Amino-3-chlorobenzoic acid (XVI) was inactive at 4 times this dosage. With other PABA analogs previously reported to have antibacterial activity, the pattern was the same: 2-substituted PABAs [2-nitro (XIV), 2-bromo (III) and 2-methyl (XI)] were found anticoccidial, while 3-substituted PABAs [3-nitro (XV), 3-methyl (XVII)] were inactive at levels tested.

In exploration of the 2-substituted PABA series more than 400 compounds have been examined. It was discovered that compounds of the general type indicated by Fig. 1a, 4-aminobenzoic acids substituted at the 2-position by a group XR, where X = O, S or NH and R = lower alkyl or alkenyl, are highly active in control of *E. maxima*. Potency in the amino acid series appears maximum in the 2-ethoxy compound which is effective against *E. maxima* at 0.0001% in diet.

Derivatives of active amino acids in which the carboxyl is esterified and/or the amino group is acylated are also potent coccidiostats. Improved feed stability is obtained by the use of such derivatives. One compound of this type, methyl 4-acetamino-2-ethoxybenzoate (Fig. 1b) has been assigned the generic name *ethopabate*. This paper reports the results of chemical and biological studies on ethopabate and congeners.

Materials and methods. Chemical. Methyl-4-amino-2-ethoxybenzoate (XX). To a stirred mixture of 10 g of 4-amino-2-ethoxybenzoic acid in 260 ml of methanol was added 26 ml of concentrated sulfuric acid. The solution was heated under reflux for 17 hours. Most of the methanol was removed *in vacuo* with the bath temperature kept below 40°. A 10% sodium carbonate solution was added with cooling until the mixture was alkaline. Methyl-4-amino-2-ethoxybenzoate separated and was recrystallized from methanol-water, m.p. 101-102°. *Anal.* Calcd. for $C_{10}H_{13}NO_3$: C, 61.54; H, 6.71; N, 7.18. Found: C, 61.82; H, 6.74; N, 6.60. *Methyl-4-acetamido-2-ethoxybenzoate (I).* Grimme(12) reported this compound as an oil. It has been obtained crystalline, and when crystallized from methanol-water melts at 149°. *Anal.* Calcd. for $C_{12}H_{15}NO_4$: C, 60.75; H, 6.37; N, 5.90. Found: C, 60.80; H, 6.41; N, 5.88. *Methyl-4-benzamido-2-ethoxybenzoate (XXII).* To a stirred solution of 1.95 g of methyl-4-amino-2-ethoxybenzoate in 80 ml of ether was added simultaneously 1.4 g of benzoyl chloride and 0.4 g of sodium hydroxide in 5 ml of water. After stirring one hour the ether was separated, washed with water, dried, and evaporated. The residue was crystallized from methanol to give methyl-4-benzamido-2-ethoxybenzoate, m.p. 127-128°. *Anal.* Calcd, for $C_{17}H_{17}NO_4$: C, 68.21; H, 5.73; N, 4.68. Found: C, 68.09; H, 5.48; N, 4.64. *4-Amino-2-ethylaminobenzoic acid (XIII).* Two and one-tenth grams of 2-ethylamino-4-nitrobenzoic acid(15) in 30 ml of alcohol containing 2 ml of concentrated hydrochloric acid was hydrogenated in the presence of 0.1 g of platinum oxide. After the theoretical amount of hydrogen was absorbed the catalyst was removed by filtration and the filtrate concentrated to dryness. The residue was dissolved in 10 ml of water containing a trace of sodium bisulfate, treated with Darco, and filtered. Sodium acetate was added to the filtrate until the solution was not acid to Congo red paper. Upon cooling, the product crystallized. It was recrystallized from 7 ml of absolute alcohol containing 1 ml of water. The compound decomposed at 130-131° and had a tendency to darken with a resulting lowering of the melting point. *Anal.* Calcd. for $C_9H_{12}N_2O_2$: C, 59.98; H, 6.71; N, 15.54. Found: C, 59.63; H, 6.79; N, 15.89. The

TABLE I. Anticoccidial Potency of 4-Amino-2-Ethoxybenzoic Acid and Related Compounds.

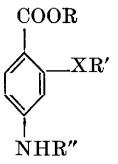
Compound	Benzene ring substituents 1	2 or 3	4	Minimum effective dose (MED), % in feed	% Reduction of oocyst count ½ MED	MED	2 MED	Synthesis reference
II	COOH	2-Cl	NH_2	0.006	32	95	100	2
III	"	2-Br	"	>0.05	—	—	—	10
IV	"	2-OH	"	>0.1	—	—	—	*
V	"	2-OCH_3	"	0.001	19	75	97	11
VI	"	2-OC_2H_5	"	0.0001	0	62	100	12
VII	"	2-OC_3H_7n	"	0.001	6	59	97	"
VIII	"	2-OC_3H_7i	"	0.001	32	75	99	"
IX	"	2-$OCH_2CH{=}CH_2$	"	0.00025	0	48	95	"
X	"	2-SC_2H_5	"	0.00025	37	98	100	13
XI	"	2-CH_3	"	0.025	0	49	97	2
XII	"	2-C_2H_5	"	0.006	0	98	—	14, *
XIII	"	2-NHC_2H_5	"	0.005	<41	74	—	*
XIV	"	2-NO_2	"	0.0125	26	47	74	16
XV	"	3-NO_2	"	>0.05	—	—	—	*
XVI	"	3-Cl	"	>0.05	—	—	—	1c
XVII	"	3-CH_3	"	>0.05	—	—	—	2
XVIII	"	3-OCH_3	"	>0.001	—	—	—	17
XIX	"	2-OC_2H_5	$NHCOCH_3$	0.0001	31	39	92	11
I	$COOCH_3$	"	"	0.00005	2	46	58	12, *
XX	"	"	NH_2	0.0001	35	79	97	*
XXI	$COOC_2H_5$	"	$NHCOCH_3$	0.00005	36	60	77	11
XXII	$COOCH_3$	"	$NHCOC_6H_5$	0.00025	<35	100	100	*
XXIII	$CONH_2$	"	NH_2	0.006	0	39	97	18
XXIV	COOH	"	NO_2	0.002	<31	70	71	19
XXV	CHO	2-OCH_3	$NHCOCH_3$	0.001	19	67	72	20
XXVI	CH_3	2-OC_2H_5	NH_2	0.0001	25	52	61	21
XXVII	"	"	NO_2	0.003	17	56	—	"

* See *Materials and methods. Chemical.*

melting point obtained for *4-amino-2-ethylbenzoic acid (XII)* was 95-97° whereas Giebe(14) reported 179-180°. The analyses, neutral equivalent and N.M.R. are all in agreement with the indicated structure. With the exception of 4-amino-salicylic acid (IV) and 4-amino-3-nitrobenzoic acid (XV), which were obtained from commercial sources, all the other compounds tested were prepared by literature methods as indicated in the references in Table I.

Biological. Assay method. Straight-run White Leghorn chicks in groups of 3 each, were weighed and placed in cages. They were fed a standard laboratory ration in which graded concentrations of test-chemicals were blended just prior to use. The normal and infected control birds were fed the basal ration. On the second day of the test the chicks were inoculated orally with 100,000 sporulated oocysts of *E. maxima*. Six days later the birds were sacrificed and weighed. Pooled small intestines were brought to a volume of 100 ml with tap water and homogenized in a blendor. Five ml of the homogenate were added to 5 ml of N NaOH for oocyst counting. Two aliquots of the suspension were placed in separate counting chambers of a hemocytometer. The oocysts in 0.5 μl of suspension were counted. The principal criterion for activity was the reduction of oocysts in the medicated groups compared to infected non-medicated control groups. The minimum concentration of the drug in the feed which reduced significantly the number of oocysts (> 38% reduction compared with controls) in the sample was determined.

Results. In Table I biological data on 27 PABA derivatives are presented. The minimum effective dose (MED) required for con-



	R	X	R'	R"
Ia	H	O, S, NH	$C_nH_{2n} \pm 1$ n = 1–4	H
Ib	CH_3	O	C_2H_5	$COCH_3$

FIG. 1. General structures for 2-substituted p-aminobenzoic acids.

trol of *E. maxima* and the percent reduction of oocyst counts at ½ MED, MED and 2 MED are tabulated. The activity of the last 10 compounds listed suggests that conversions to a common active product, presumably 4-amino-2-ethoxybenzoic acid, are very efficient metabolic operations. In tests against other intestinal species of coccidia, these compounds were found to be of approximately equal effectiveness. Ethopabate was studied most intensively. Most laboratory strains of *E. maxima* are controlled by ethopabate at feed levels of 0.0004-0.0008%. Similar results are observed in the less thoroughly studied cases of *E. brunetti* and *E. acervulina*. Ethopabate and related compounds are inactive against *E. tenella* at 0.01%.

The anticoccidial activity of 4-amino-2-ethoxybenzoic acid is antagonized by the simultaneous administration of an equal weight of PABA. It is of interest to note also that 4-amino-2-ethoxybenzoic acid potentiates the anticoccidial action of pyrimethamine.

2-Chloro-PABA, like the 2-ethoxy compound, is overcome by PABA. Methionine, which has been observed to reverse 2-chloro-PABA in some bacterial systems(1a,7), does not affect its anticoccidial activity.

In cell-free extracts of *E. coli,* the following sequence of reactions is postulated for tetrahydrofolate synthesis(8):

A. 2-Amino-4-hydroxy-5-hydroxymethyldihydropteridine + PABA $\xrightarrow[Mg^{++}]{ATP}$ Dihydropteroic Acid

B. Dihydropteroic Acid + Glutamic Acid ⟶ Dihydrofolic Acid

C. Dihydrofolic Acid + 2H ⟶ Tetrahydrofolic Acid

Sulfanilamide primarily blocks reaction A above. The sulfanilamide analog of dihydropteroic acid, N^4-(2-amino-4-hydroxy-6-dihydropteridinylmethyl)-sulfanilamide, is formed to some extent and possibly exerts secondary effects on reactions A and B. PAS is similarly incorporated(9) and, probably, 4-amino-2-ethoxybenzoic acid may also replace PABA with consequent formation of 2′-ethoxydihydropteroic acid. Is the anticoccidial action due to inhibition of reaction A by 4-amino-2-ethoxybenzoic acid or inhibition of reaction B by 2′-ethoxydihydropteroic acid? This question assumes, of course, that the biochemical pathway of tetrahydrofolate synthesis in coccidia is like that found in *E. coli.* On this assumption, two arguments favor the reaction B inhibition mechanism or inhibition of both reactions. The effect of an *o*-ethoxy group in PABA is most reasonably interpreted as a *steric* effect upon reaction B, the enzymatic coupling of the dihydropteroic acid carboxyl with glutamic acid. The fact that 4-amino-2-ethoxybenzoic acid does not inhibit growth of sulfa-sensitive bacteria suggests that reaction A is not involved.

Summary. 1. Preparations of the 4-amino, 4-acetamido and 4-benzamido derivatives of methyl-2-ethoxybenzoate and of 4-amino-2-ethylaminobenzoic acid are reported. 2. Anticoccidial activity is demonstrated for 2-substituted PABAs, especially those containing 2-alkoxy, alkythio and alkylamino groups. 3. The most potent compounds are 4-amino-2-ethoxybenzoic acid and its ester and N-acyl derivatives.

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Alterations in Circulating Folate Induced by B_{12} Deficiency.* (29617)

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Folate metabolism is abnormal in frank vit. B_{12} deficiency(1-5). This report deals with the fate of ingested synthetic pteroylglutamic acid (PGA) during a B_{12} deficiency as compared with a folate deficiency. The investigation was undertaken to learn more about circulating folates in these deficiency states.

There has been much conjecture about the nature of the circulating folates in B_{12} deficiency. To identify them, serum was chromatographed and activities charted by bioautography with 3 folate-requiring bacteria whose known differential responses permit a closer identification of folates.

Materials and methods. Lactobacillus casei (ATCC 7469), *Pediococcus cerevisiae* (ATCC 8081), and *Streptococcus faecalis* (ATCC 8043) have different responses to folates(6, 7) (Fig. 1). Assays with *S. faecalis* and *P. cerevisiae* were carried out as described for *L. casei*(8); *L. casei* basal medium was used for *S. faecalis*. *Ochromonas malhamensis* served to assay serum B_{12}(8).

An oral loading test with 5 mg of PGA was carried out as before(8,9); 15 subjects with a normal serum folate and B_{12}; 6 with normal folate and low B_{12}, and 6 with low folate and normal B_{12} were used for this study. Folate and B_{12} deficiencies were due to nutritional defects except for one case of pernicious anemia.

Serum, prepared for assay with ascorbate and buffer(8), was concentrated 50 times; non-PGA and 2-hour PGA loaded serum was used. One-tenth ml of the concentrate was spotted and dried on Whatman no. 1 paper (4 × 30 cm). Saturated Na_2HPO_4 was used as a solvent system for the 5 hour ascending chromatography; afterward the paper was dried at 55°C. Following chromatography, bioautography with the 3 organisms was employed to identify folates. A mixture of 300 ml of basal medium together with 6 g of agar was autoclaved and cooled to 45°C; 225 mg of tetrazolium red was added aseptically. Tetrazolium red becomes bright red on reduction and helps define growth spots. The bioautography medium, inoculated with 1 ml of organism grown for 18 hours in

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