[CONTRIBUTION FROM THE LABORATORY OF BIOCHEMISTRY, NATIONAL CANCER INSTITUTE, NATIONAL INSTITUTES OF HEALTH, PUBLIC HEALTH SERVICE]

Synthesis of Allothreonine–Threonine from Acetaldehyde and Glycine by a Rat Liver Enzyme[1]

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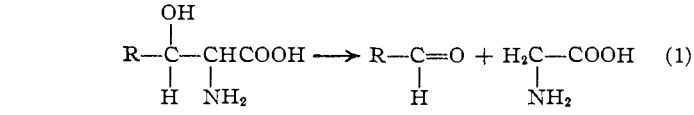
Addition of acetaldehyde and radioactive glycine to a buffered (*p*H 7.9) aqueous solution of a rat liver fraction followed by incubation at 37° resulted in the formation of radioactive allothreonine–threonine.[3] This β-hydroxy-α-amino acid[3] was not detectable when the enzyme was omitted from the digest. Separation of the radioactive products of the reaction was achieved on a cation-exchange resin column. The identification of allothreonine–threonine was accomplished by chromatography of the amino acid and the dinitrophenyl derivative of the amino acid with the respective authentic compounds. Additional evidence was obtained through recrystallization to constant specific activity with carrier DL-allothreonine, and by degradation with periodate. It is probable that the predominant end product of the reaction was one or both isomers of allothreonine. The significance of this finding in relation to intermediary metabolism is discussed.

I. Introduction

A relationship between glycine and β-hydroxy-α-amino acid metabolism was first stated by Knoop.[4] The hypothesis that β-phenylserine was cleaved *in vivo* to benzoic acid and glycine was made on the evidence of the isolation of hippuric acid as the excretory product of this β-hydroxy-α-amino acid. Considerably later, Braunshtein[5] described the enzymatic conversion of several β-hydroxy-α-amino acids, including threonine but not serine,[6] to glycine and the corresponding aldehyde.



(1)

The reaction consisted of cleavage of the bond between the second and third carbon atoms; *e.g.*, β-phenylserine yielded glycine and benzaldehyde as end products.[7] The same reaction was also observed non-enzymatically. For example, in strongly alkaline solutions, β-hydroxy-α-amino acids yielded glycine.[8,9]

The interesting and suggestive finding that a reversible splitting of threonine occurred in the presence of pyridoxal and certain metal ions in aqueous solution was described by Metzler, Longenecker and Snell.[10] It therefore appeared of interest to examine the reversibility of the reaction, eq. 1, when the enzyme was the catalyst. Indeed, this might well be the synthetic route suggested by the work from several sources[11–13] that demonstrated that glycine was a precursor of threonine in yeast. This enzyme which has been variously termed "glycinogenase"[5] and "threonine aldolase"[14] has been shown to be present in liver and kidney tissue of numerous mammals and the pigeon,[5] and probably in Neurospora.[15,16]

II. Experimental

Enzymatic Digests.—The rat liver fractions employed were prepared as previously described.[7] Activities of the three enzyme preparations used were expressed as 20, 26 and 33 μmoles benzaldehyde/hr./mg. N, with *erythro*-β-phenyl-DL-serine as substrate under conditions previously specified.[7] The reaction mixtures consisted of 200 μmoles of glycine plus a trace amount of glycine-2-C^{14} or glycine-1-C^{14}, 200 μmoles of acetaldehyde, and 10 mg. of the purified rat liver enzyme in a volume of 5 ml. of which 3 ml. were 0.15 *M* borate buffer, *p*H 8.0. The digests were incubated at 37° under aerobic conditions with agitation for 4 hours. The final *p*H of the digests was 7.9. The reaction was stopped by addition of 1 ml. of a 5% solution of trichloroacetic acid (aqueous). After precipitation of the protein, the solutions were lightly centrifuged and the supernatant decanted. Two types of controls accompanied the above, consisting of (1) enzyme (10 mg.) plus 3 ml. of borate buffer plus a trace of labeled glycine and (2) 200 μmoles of acetaldehyde and glycine each, plus a tracer amount of radioactive glycine.

Compounds.—Glycine-1-C^{14} and glycine-2-C^{14} were commercially obtained (Nuclear Instrument and Chemical, and Tracerlab, Inc., respectively). They were purified by Dowex 50 column chromatography followed by unidimensional paper chromatography in "formix" followed by elution. After these procedures, the purity was tested by two-dimensional paper chromatography. A radioautograph and a test of the chromatogram for ninhydrin positive areas were made. No spots other than that of glycine were present. Threonine-C^{14} (uniformly labeled) was a Schwarz Laboratory product. The specific activities of the three radioactive amino acids were 39, 18 and 0.8 μC./mg., respectively. The glycine, DL-threonine and DL-allothreonine used were recrystallized commercial compounds with correct analytical values. Dinitrophenyl (DNP) derivatives of several amino acids were kindly supplied by Dr. H. Sober.[17]

Chromatographic Materials and Technique.—Dowex 50 (200 mesh) was used in a column measuring 2.2 × 50 cm.

(1) The subject matter of this paper was presented at the 127th National Meeting of the American Chemical Society at Cincinnati, Ohio, March, 1955, Abstracts of Papers, p. 26-C.

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(3) Allothreonine–threonine will be employed as the designation for any one or more of the four possible optical isomers of α-amino-β-hydroxybutyric acid without distinction.

(4) F. Knoop, *Z. physiol. Chem.*, **89**, 151 (1914).

(5) A. E. Braunshtein and G. Ia. Vilenkina, *Doklady Akad. Nauk S.S.S.R.*, **66**, 243 (1949); G. Ia. Vilenkina, *ibid.*, **84**, 559 (1952).

(6) The enzyme as so far characterized also has been shown to have no activity with respect to α-methylserine (see reference in footnote 7).

(7) J. B. Gilbert, THIS JOURNAL, **76**, 4183 (1954).

(8) T. Wieland and L. Wirth, *Ber.*, **82**, 468 (1949).

(9) J. M. Bremner, *Nature*, **168**, 518 (1951).

(10) D. E. Metzler, J. B. Longenecker and E. E. Snell, THIS JOURNAL, **76**, 639 (1954)

(11) F. Kogl and W. A. F. Borg, *Z. physiol. Chem.*, **269**, 97 (1941).

(12) I. A. Rossi and C. Cennamo, *Boll. soc. ital. biol. sper.*, **20**, 194 (1945).

(13) A. R. Fanelli, *Farmaco sci. e tec. (Pavia)*, **1**, 6 (1946).

(14) S. C. Lin and D. M. Greenberg, *J. Gen. Physiol.* **38**, 181 (1954). Note also the recent report, M. A. Karasek and D. M. Greenberg, *Federation Proc.*, **15**, 284 (1956), that sheep liver contained two enzymes, one cleaving L-allothreonine, and the other L-threonine. The enzyme active with respect to the former compound was activated by pyridoxal phosphate.

(15) R. P. Wagner and A. Bergquist, *J. Biol. Chem.*, **216**, 251 (1955).

(16) A preliminary report indicated the presence of the enzyme in *Tetrahymena pyriformis*. V. C. Dewey and G. W. Kidder, Resumes of Communications, International Congress of Biochemistry, 3rd, Brussels, 1955, p. 92.

(17) K. R. Rao and H. A. Sober, THIS JOURNAL, **76**, 1325 (1954).

at a flow rate of 7.5 ml./hr. Size of fractions cut was approximately 9–10 ml.; 1.5 *N* HCl was used as the eluting agent throughout.[18]

All two-dimensional chromatograms employed 17 in. squares of Whatman no. 1 paper. Unidimensional chromatograms were either on this size paper or on paper strips 1/4 in. wide and 15 in. long. Solvents employed were (a) for amino acids (1) phenol, H_2O (the phenol was saturated with respect to a 10% sodium citrate aqueous solution) followed by (2) formic acid, *t*-butyl alcohol, H_2O ("formix") (volume proportions 15:70:15); (b) for DNP-amino acids (1) toluene, chloroethanol, pyridine, 0.8 *N* NH_3 (5:3:1.5:3)[19] (2) 1.5 *M* phosphate (*p*H 6).[19] The dinitrophenyl derivative of enzymatically synthesized allothreonine–threonine was prepared by the procedure of Sanger.[20]

Assay of Radioactivity.—Counting of radioactive samples was accomplished on a continuous-flow windowless counter using helium (98.7%)-butane (1.3%) as the self-quenching gas mixture. Only aluminum planchets were employed for $BaCO_3$ samples. All other samples were plated directly on ground glass or aluminum planchets. Where measurement of low activities was important, *e.g.*, Fig. 4, a Robinson counter with background of 3.8–4.0 c.p.m. was employed. Counting was for such a period of time as to make the standard error 5% or less on the corrected sample measurements.

Degradation of Threonine.—Allothreonine–threonine was degraded by the periodate method[21] with some modification. The periodate oxidation flask was connected to two ice-cold bubblers containing 0.1% 2,4-dinitrophenylhydrazine in 2 *N* HCl, followed by a bubbler containing 0.5 *N* sodium hydroxide. The acetaldehyde, as the 2,4-dinitrophenylhydrazone, was filtered, plated and counted. Carbonate from the sodium hydroxide bubbler was precipitated by addition of ammonium chloride and $BaCl_2$.[22]

III. Results

After removal of the protein, an aliquot of the enzymatic digest containing acetaldehyde and glycine-C^{14} was chromatographed in two dimensions (Fig. 1) with the result that a ninhydrin positive, radioactive spot (labeled B) was observed in addition to the glycine area (A). No such spot appeared in a control from which enzyme had been excluded.

The protein-free reaction mixtures, originally incubated with and without enzyme added to the initial incubation mixtures, were placed on a Dowex 50 resin column and eluted with 1.5 *N* HCl. The results (Fig. 2) indicated that where enzyme was originally present, two peaks appeared at 340 and 360 ml., respectively. When the rat liver fraction was absent, only the peak at 360 ml. was present. The two peaks at 360 ml. contained approximately equal amounts of radioactivity. The radioactivity contained in the 490–600 ml. fractions represented glycine.

The several fractions representing the peaks at 340 and 360 ml. were combined and chromatographed in two dimensions with the result shown in Fig. 3. It was noted that when carrier DL-threonine was added, the radioactivity was congruent with this spot when enzyme was present in the incubation mixture. In the other chromatogram, where no enzyme was added, no radioactivity was present; only the ninhydrin positive carrier, DL-threonine area, was demonstrable.[23] The identity of the second component was not ascertained. However, it was present predominantly

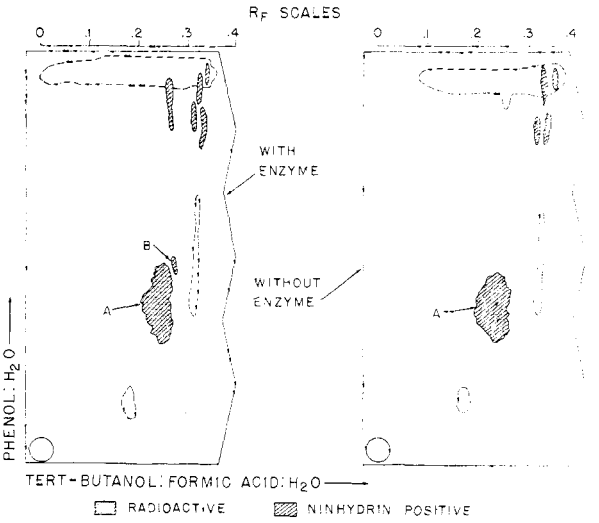

Fig. 1.—Two-dimensional paper chromatograms of aliquots from reaction mixtures of glycine-2-C^{14} and acetaldehyde, with and without enzyme added. Areas of color development following application of ninhydrin are indicated as well as optical densities on a coincident radioautograph. The origin is designated by the circle at the lower left corner in both instances. The chromatograms have been interrupted in the *t*-butyl alcohol:formic acid:H_2O dimension and R_f scales substituted: A, glycine; B, area suggesting synthesis of allothreonine–threonine; radioactive areas in upper right portion are unidentified; areas enclosed by dashed line represent light radioactive or ninhydrin positive areas which may be ignored.

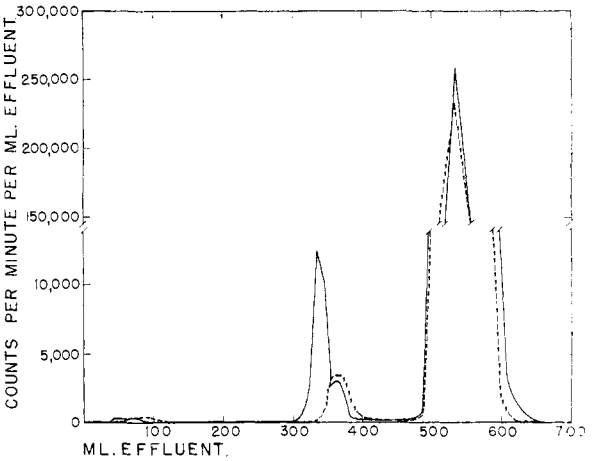

Fig. 2.—Separation on Dowex 50 column of products of reaction of acetaldehyde with glycine-1-C^{14}, 1.5 *N* HCl employed as eluant: ——, original incubation mixture contained enzyme; – – –, enzyme absent from the initial digest.

(18) W. H. Stein and S. Moore, *Cold Spring Harbor Symposia Quant. Biol.*, **14**, 179 (1949).

(19) A. L. Levy, *Nature*, **174**, 126 (1954).

(20) F. Sanger, *Biochem. J.*, **45**, 563 (1949).

(21) A. M. Delluva, *Arch. Biochem. Biophys.*, **45**, 443 (1953).

(22) M. Calvin, C. Heidelberger, J. C. Reid, B. M. Tolbert and P. E. Yankwich, "Isotopic Carbon," John Wiley and Sons, Inc., New York, N. Y., 1949, p. 85.

(23) The second type of control, which contained enzyme and radioactive glycine only, was also subjected to column and paper chromatography. Essentially no radioactivity was found in the 300–400 ml. interval from the cation-exchange resin column. One-third of this interval volume was concentrated and examined by two-dimensional paper chromatography. No ninhydrin color was located in the threonine area. It was concluded that the enzyme was a negligible source of threonine, if it contributed any at all.

in the 360 ml. peak and was essentially absent from the early fractions of the 340 ml. peak.

The area marked threonine on the left chromatogram of Fig. 3 was eluted and reacted with 1-

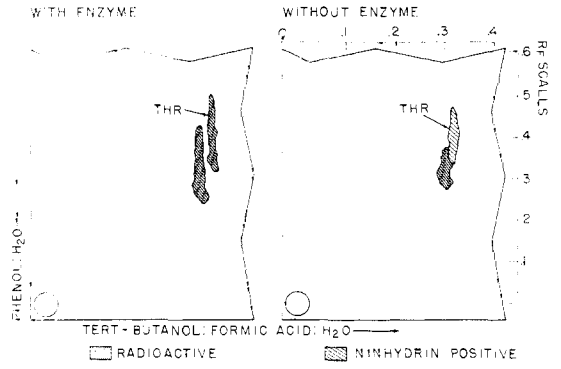

Fig. 3.—Two-dimensional paper chromatograms of aliquots from the radioactive fractions eluted from Dowex-50 column in the interval 300 to 400 ml., inclusive (*cf.* Fig. 2). Carrier DL-threonine added and location indicated (THR). Conventions as in Fig. 1 except that chromatograms have been interrupted in both solvent dimensions and approximate R_f scales substituted. The "with enzyme" chromatogram is drawn to the same R_f scale as the chromatogram on the right.

fluoro-2,4-dinitrobenzene. The resulting dinitrophenyl derivative was chromatographed unidimensionally in two solvent systems: toluene, chloroethanol, pyridine, NH_3 and phosphate buffer. Coincidence of DNP-"threonine" with authentic DNP-DL-threonine was apparent when the toluene, chloroethanol, pyridine, NH_3 mixture was employed (Fig. 4). The figure presents the radioactivity and optical density at 360 mμ of several aqueous eluates from the several segments into which the chromatogram was divided. Reasonable congruency of the radioactivity curve and the optical density curves was noted. The sole exception was with respect to the dinitrophenol.

Suspected DNP–allothreonine–threonine plus carrier DNP-DL-threonine were also chromatographed using a 1.5 *M* phosphate buffer (Fig. 5). Congruency of yellow color and radioactivity was observed. Di-DNP-cystine was chromatographed since it and glycine are the common amino acids having R_f values close to DNP-DL-threonine in the solvent mixture containing toluene (Fig. 4).

The radioactivity corresponding to allothreonine–threonine was eluted from a paper chromatogram and recrystallized with DL-threonine. This was effected by addition of absolute alcohol to an aqueous solution of the amino acid. The result was: original solution, 44 c.p.m./mg. DL-threonine; first crystallization, 37; third, 30; sixth, 22; ninth, 17. When eluted allothreonine–threonine was recrystallized with DL-allothreonine, the result was: original solution, 118 c.p.m./mg. DL-allothreonine; third crystallization, 108; fifth, 110; sixth, 105.

Degradation of allothreonine–threonine-1-C^{14}, *i.e.*, initial substrate glycine-1-C^{14}, together with carrier by the periodate method yielded the following data: % of activity of original allothreonine–threonine in C-1, 70%; C-2, < 1%; C-3,4,<1%.[24] This localization of the isotope is compatible with

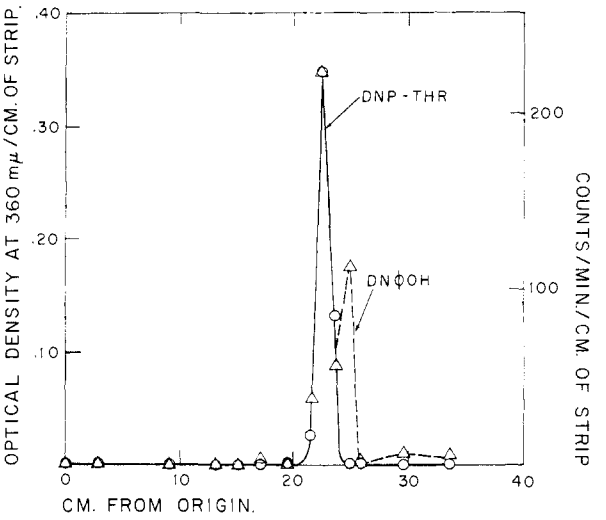

Fig. 4.—Radioactivity and yellow color recorded as a function of distance along a unidimensional paper chromatogram to which was added DNP-"threonine" and authentic carrier DNP-DL-threonine, prior to development in the toluene, chloroethanol, pyridine, NH_3 solvent system. DNP-"threonine" = a fraction from an enzymatic digest which was purified and tentatively identified as allothreonine–threonine by the procedures indicated in Figs. 2 and 3, and which reacted with fluorodinitrobenzene. DNϕOH, dinitrophenol: △- - -△, optical density; O——O, radioactivity. Chromatogram divided into segments prior to elution and measurement of data. Abscissa points represent distance of midpoint of each segment from origin.

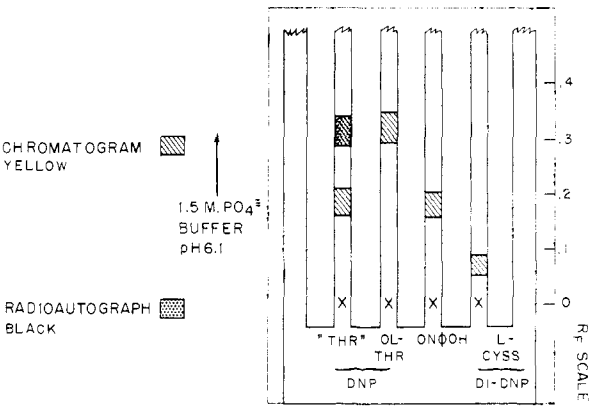

Fig. 5.—Unidimensional chromatogram of several DNP-derivatives: developing solvent, 1.5 *M* phosphate buffer, *p*H 6.1. Except for DNP-"THR," the compounds were authentic. DI-DNP-L-CYSS, di-DNP derivative of L-cystine. Other compounds identified by convention of Fig. 4. Origin on each of the narrow strips is at X.

(24) The recovery of radioactivity of carbons one and two of threonine was somewhat low in our experience. Yield was approximately 70% whereas Delluva[21] reported a yield of 85% for carbon two but made no mention of recovery of carbon one. For example, L-threonine-C^{14} (uniformly labeled—checked by specific activity determination by this periodate method, and stated in arbitrary units: C-1 0.68; C-2, 0.66; C-3,4, 0.76) yielded a recovery of 72% in C-1 and 69% in C-2. However, yield is adequate to prove localization.

the reaction proceeding as a simple reversal of eq. 1. More devious routes might well introduce randomization.

The conclusion reached from these results is that the predominant product of this enzymatic reaction *in vitro* is an isomer or isomers of DL-allothreonine. Indeed, this may be the only product. However DL-threonine (or L- or D-threonine) could be present in rather large proportion and not be detected. The specific stereoisomer(s) produced awaits future recrystallization, microbiological experiments, etc., in order to determine its configuration.

IV. Discussion

Inasmuch as allothreonine has not been identified in biological material, it obviously is impossible to relate this compound to present schemes of intermediary metabolism. The present findings do suggest the possible natural occurrence of this diastereoisomer, however.[25]

It is worth reiterating that no serious attempt has been made in this present work to identify D- or L-threonine except as a large component. The compound might well escape detection if present in less than approximately 30% of the total amount of threonine. Indeed suggestive of the probability of this occurrence, but in lower forms of life, is the previously cited evidence obtained in yeast.[11-13,26]

(25) Relative to metabolic utilization, if not metabolic occurrence, D-allothreonine was used by certain mutant microörganisms in the biosynthesis of isoleucine and threonine [H. E. Umbarger and E. A. Adelberg, *J. Biol. Chem.*, **192**, 883 (1951)]. Therefore, this enzyme present in certain lower forms may well serve the production of a precursor of certain amino acids in such microörganisms. However, in the rat it is well established that only one isomer, L-threonine, is utilized for growth purposes [H. D. West and H. E. Carter, *ibid.*, **122**, 611 (1938)].

(26) A more remote possibility is suggested by a *Neurospora crassa* mutant which revealed properties which may be explained by an alternate metabolic pathway for the synthesis of threonine [E. A. Adelberg, C. A. Coughlin and R. W. Barratt, *J. Biol. Chem.*, **216**, 425 (1955)]. This is suggested inasmuch as this double mutant was blocked as follows: homoserine ⊣→ threonine ⟶ α,β-dihydroxy-β-ethylbutyric acid ⊣→ α-keto-β-ethylbutyric acid. It, in minimal medium, synthesized threonine at a slow rate. That one strain of this species, at least, has the enzyme in question, has been reasonably well authenticated by Wagner and Bergquist (footnote 15). However, glycine did not stimulate production of α,β-dihydroxy-β-ethylbutyric acid. Compare also discussions of the possibility of L-threonine synthesis by this condensation reaction in *Escherichia coli* [J. O. Meinhart and S. Simmonds, *J. Biol. Chem.*, **213**, 329 (1955)] and in *Clostridium kluyveri* [N. Tomlinson, *ibid.*, **209**, 597 (1954)].

Also suggestive is the fact that L-allothreonine was cleaved at approximately seven times the rate that L-threonine was split by the enzyme concerned[5] (prepared from rat liver).[14] So it may well be true that the kinetics of the synthetic reaction, favors allothreonine formation in some such proportion. Furthermore, in the corresponding reversible model reaction[10] both threonine and allothreonine are produced and in approximately comparable amounts. Since both are pyridoxal catalyzed, a somewhat similar relationship should be expected for the enzymatic reaction.

The essentiality of threonine for mammals implies that synthesis of L-threonine by such a route, if it does indeed occur, is of a relatively small quantity compared to the daily growth requirement. Perhaps the availability of acetaldehyde may limit the utility of this reaction in animals. By the same token in yeast, the reaction may be of importance as a synthetic mechanism because acetaldehyde is a major metabolite.

Acknowledgment.—The author wishes to thank Dr. J. P. Greenstein for his interest, Drs. H. Sober and E. Peterson for their advice on chromatography, Mr. R. J. Koegel for the elemental analyses, and Dr. M. Calvin for the opportunity to acquire experience in radioactive isotopic procedure some time ago.

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Some New *o*-Phenylenediamines and the Related Benzimidazoles, Benzotriazoles and Quinoxalines

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The synthesis of 3,4-diamino-5-nitrophenetole and 3,4-diamino-2-nitroanisole is described. These *o*-diamines have been used to prepare the related benzimidazoles, benzotriazoles and quinoxalines which were needed to continue an investigation of the inhibition of developing *R. pipiens* embryos by such heterocyclic compounds. Derivatives of 3,4-diamino-2-nitroanisole have served as useful reference compounds for the solution of several problems of structure.

Among a number of benzimidazoles, benzotriazoles and quinoxalines previously reported,[1,2] several have shown interesting properties as inhibitors of developing *R. pipiens* embryos. The most active of these were 4-methoxy-6-nitrobenzimidazole, 4-methoxy-6-nitrobenzotriazole and 5-methoxy-7-nitroquinoxaline.[3] Isomers of these heterocyclic compounds, in which the nitro and methoxy groups were interchanged, exhibited an approximately equal level of activity.[4] It seemed of interest, therefore, to investigate whether the activity of such compounds might be enhanced or diminished by changing the methoxy group to ethoxy and changing the relative configuration of the nitro and methoxy groups to *ortho* rather than *meta*. The synthesis of the desired substances required the preparation of two new substituted *o*-phenyl-

(1) H. Gillespie, M. Engelman and S. Graff, THIS JOURNAL, **76**, 3531 (1954).

(2) H. Gillespie, M. Engelman and S. Graff, *ibid.*, **78**, 2445 (1956).

(3) K. Liedke, M. Engelman and S. Graff, *J. Exp. Zool.*, **127**, 201 (1954).

(4) K. Liedke, private communication.