Induction of D-amino acid oxidase in germ-free mice
by Leon Richards Lyle

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY in Microbiology
Montana State University

Abstract:
It has long been known that the enzyme, D-amino acid oxidase (E.C. 1.4.3.3) is present in the tissues and organs of various mammals. It was observed, by oxygen recording polarography, that the enzyme activity was apparently absent from the kidneys and macrophages of germ-free Swiss mice. In contrast, the enzyme was present in the kidneys and macrophages of conventionally reared mice, raising the question of whether or not D-amino acid oxidase is an inducible enzyme in the Swiss mouse. Boiled preparations from conventionally reared mouse kidneys were inactive. Little or no activity was demonstrable when L-alanine was substituted for D-alanine in the assay system. D-amino acid oxidase activity was not present in the kidneys of a fetal human or in the kidneys of conventionally reared mice less than twenty four hours of age. However, neonatal conventionally reared mice acquired adult levels of the enzyme during the first two weeks of life. Germ-free mice could be stimulated to produce the enzyme by administration of D-amino acids, exposure to living gram-positive microorganisms, inactivated gram-positive microorganisms, and preparations derived from their cell walls. The rapid passage of 14C-(U) D-alanine labelled Bacillus cereus cell wall material across the gastrointestinal mucosa of the germ-free mouse was observed, and the decline in the amount of radioactive material in their kidneys was accompanied by a significant increase in the levels of D-amino acid oxidase. In summary, these results suggest that the normal bacterial flora of the gastrointestinal tract may play an important role in the induction of host enzymes.

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Abstract

It has long been known that the enzyme, D-amino acid oxidase (E.C. 1.4.3.3) is present in the tissues and organs of various mammals. It was observed, by oxygen recording polarography, that the enzyme activity was apparently absent from the kidneys and macrophages of germ-free Swiss mice. In contrast, the enzyme was present in the kidneys and macrophages of conventionally reared mice, raising the question of whether or not D-amino acid oxidase is an inducible enzyme in the Swiss mouse. Boiled preparations from conventionally reared mouse kidneys were inactive. Little or no activity was demonstrable when L-alanine was substituted for D-alanine in the assay system. D-amino acid oxidase activity was not present in the kidneys of a fetal human or in the kidneys of conventionally reared mice less than twenty four hours of age. However, neonatal conventionally reared mice acquired adult levels of the enzyme during the first two weeks of life. Germ-free mice could be stimulated to produce the enzyme by administration of D-amino acids, exposure to living gram-positive microorganisms, inactivated gram-positive microorganisms, and preparations derived from their cell walls. The rapid passage of ^{14}C-(U) D-alanine labelled Bacillus cereus cell wall material across the gastrointestinal mucosa of the germ-free mouse was observed, and the decline in the amount of radioactive material in their kidneys was accompanied by a significant increase in the levels of D-amino acid oxidase. In summary, these results suggest that the normal bacterial flora of the gastrointestinal tract may play an important role in the induction of host enzymes.

INTRODUCTION

The preponderance of L-amino acids in the biological environment is well recognized, (Conn and Stumpf, 1966). This is the case both with respect to amino acids incorporated into protein and those free amino acids that are accumulated or participate in some other way in metabolic events. The apparent status of the enantiomorphic counterpart, the D-amino acid, is that of a biological pariah. This situation is somewhat enigmatic in that Miller (1955) has shown that the amino acids formed by the action of ultraviolet light and electrical discharges on an ammonia, methane and hydrogen mixture, over water, were either symmetric (β-alanine) or if asymmetric, occurred in equimolar quantities having no net optical activity. Asymmetric synthesis of amino acids using non-enzyme catalyzed reactions have been accomplished, however, and presumably occurred on the primitive earth, (Harada, K., 1963, Harada, K., 1965, and Harada, K. 1966). Further speculations along these lines are discussed by Gardner (1969).

In contrast to the ubiquity of L-amino acids in biological systems, D-amino acids are associated, as structural components, only with the cell walls of microorganisms, (Martin, 1966). However, one D-amino acid is present (presumably transiently) in the serum of rodents, (Hoeprich, 1965). These considerations, taken with the observation that the enzyme D-amino acid oxidase (E.C. 1.4.3.3)

occurs in mammalian tissue, (Krebs, 1935), raise interesting questions as to the origin and functional significance of mammalian D-amino acid oxidase.

With regard to the origin of mammalian D-amino acids, one author has made the observation that the sera of conventionally reared (CR) mice contains approximately 0.30 μmoles of D-alanine per ml, whereas the sera of germ-free (GF) mice contains no detectable levels of D-alanine, (Hoeprich, 1965). The obvious implication is that the presence or activities of the normal microbial flora contribute to these observations.

The physiological significance of D-amino acid oxidase in mammalian tissues has remained a mystery for approximately thirty years. As late as 1960 one apparently frustrated author was prompted to issue the "central dogma" of D-amino acid oxidase viz. "The mere demonstration of the existence of an enzyme activity does not, of course, mean that it is of physiological significance," (Meister, 1960). This situation has been improved somewhat with the observation that D-amino acid oxidase functions as a terminal respiration system in mammalian cells, (de Duve, C. and Baudhuin, 1966). Also, it has very recently been shown that the D-amino acid oxidase of human and guinea pig leucocytes is capable of deaminating D-amino acids as they reside <u>in situ</u> on bacterial cell walls thus enhancing

the bacteriolytic activities of the polymorphonuclear leucocyte and the macrophage, (Cline, M.J., and R.I. Lehrer, 1969).

There is something of a discrepancy, however, with respect to the organs of various rodents in which D-amino acid oxidase may be found. Cline and Lehrer (1969) report D-amino acid oxidase in the leucocytes of humans and in the leucocytes, spleen, kidney, and liver of the CR guinea pig. Meister et al. (1960) found apparently equal concentrations of the enzyme in the kidneys of adult CR and GF mice. Shack (1943), on the other hand, was unable to detect the enzyme in the livers of CR mice but reported its presence in the CR rat liver and kidney. According to Meister and co-workers (1960), the D-amino acid oxidase activity in the kidneys and livers of newborn rats is about 10% of the maximal activity achieved at 25 to 35 days of age. The same authors reported that injection or oral administration of D-amino acids to very young rats does not affect the normal course of development of D-amino acid oxidase activity. This finding seems to discount the possibility that induction of the enzyme results from contact with the substrate in the environment shortly after birth. However, it has been shown that whereas the injection of small doses of tryptophan into very young rats increases the activity of tryptophan pyrrolase during the period from 7 to 10 days of age to adulthood, large doses of the amino acid

decrease the activity of the enzyme, a phenomenon likened to immunological tolerance produced by large doses of antigen, (Van Bekkum and Nieuwerkerk, 1965).

As the question of the inducible nature of D-amino acid oxidase remains unresolved, it is proposed that this question be reexamined by comparative studies in GF and CR mice. This report presents evidence, derived from multiple lines, that D-amino acid oxidase synthesis is induced by substrates provided by the Gram-positive component of the normal microbial flora.

MATERIALS AND METHODS

Experimental animals. The Swiss mice used in these studies were originally obtained from Manor Farms at Staatsburg, New York in 1964. Since then they have been maintained by random colony breeding in both conventional and axenic environments. All mice were housed in plastic cages in either a conventional environment or Trexler flexible film isolators and received water and food materials ad libitum. Conventionally reared (CR) mice were fed Purina Mouse Chow and tap water. Germ-free (GF) mice received sterile distilled water and Purina #5010 C mouse chow which had been autoclaved for 35 minutes at 15 pounds pressure and dried under vacuum. Other maintenance procedures employed have been previously described by Reed and Jutila (1967) and Reed (1966).

Feed. Purina Laboratory Chow #5010 C is an enriched formula designed to contain after autoclaving, levels of nutrients comparable to non-autoclaved mouse chow. It is prepared under hygienic conditions and contains less than 1000 organisms per gram of chow. These consist of Streptococcus sp., Staphylococcus sp., Gram-positive aerobic and anaerobic bacilli, yeast and molds, (Damou, 1969). Because of quantitative differences in the nutrient value of laboratory mouse chow before and after autoclaving, administration of #5010 C to conventionally reared mice would not constitute an appropriate control, and administration of autoclaved #5010 C to

conventionally reared mice is prohibitively expensive.

General Methods

Enzyme Isolation from Kidney and Liver Tissues. Mice were sacrificed by cervical dislocation and the tissues and organs were removed surgically. The organs were minced in ten volumes of cold 0.25M aqueous sucrose solution buffered to pH 7.2 with 0.01M phosphate buffered saline (PBS). The cells were disrupted either in a prechilled size A tissue homogenizer (No. 4288-B, Arthur Thomas Co., Philadelphia, Pennsylvania) or in a prechilled Sorvall OM-1150/MA microhomogenizer operated in the cold at 50,000 revolutions per minute for two to four minutes. Cellular lysis was verified microscopically. The homogenate was diluted in 2.7 volumes of cold buffered sucrose and was centrifuged for 30 minutes 20,000 x g in a Beckman Spinco model L-2 ultracentrifuge using the Ti-50 rotor. After the protein concentration of the preparation was estimated by the method of Lowry et al., (1951) the supernatant fluid was employed for determinations of enzyme activity.

Enzyme Isolation from Peritoneal Macrophages. Cell populations rich in peritoneal macrophage were collected from CR and GF mouse populations. Additionally, macrophages were harvested from a population

monocontaminated with Bacillus circulans. One week prior to collection, the mice received an injection of 3.0 ml of sterile thioglycollate broth. The macrophages were washed from the peritoneal cavity with 10.0 mls of Hanks balanced salts solution (BSS), injected intraperitoneally, and withdrawn with a 10.0 ml syringe. The preparations from a minimum of 10 animals for each of the three groups described above were pooled and centrifuged in the cold at 6000 rpm for 10 minutes. The cells were washed three times in cold Hanks BSS and the final packed cell mass was disrupted in a prechilled Sorvall OM-1150/MA microhomogenizer as described above in conjunction with freeze-thaw techniques. Subsequent handling of these preparations was the same as that previously described for kidney preparations.

Enzyme Activity. Estimates of enzyme activity were obtained polarographically with a Gilson Medical Electronics Model KM Oxygraph, Strobel (1966). The determinations were obtained at 25°C in accordance with the recommendations of the International Union of Biochemistry Commission of Enzymes, (I.U.B.E.C., 1961). A thermostatically controlled water bath which pumped circulating water to the water jacket surrounding the reaction chamber was used to insure this condition. The reaction mixture contained 150 μmoles of sodium pyrophosphate buffer pH 8.3, enzyme preparation, and 50.0 μmoles of D-alanine, and H_2O to a final volume of 2.5 ml, as modified from

Boulanger and Osteux (1963), Meister et al. (1960), and Strobel (1966). Additionally, each preparation contained 17.6 µg (Meister et al., 1960) of flavine adenine dinucleotide (Schwartz BioResearch, Inc., Orangburg, New York) unless otherwise noted. Under the conditions of this assay the addition of catalase was not held to be necessary (Dr. P.K. Stumpf, University of California, Personal communication). The concentration of oxygen in solution was taken as 218 mµ M/ml for purposes of calculation (Dr. Gary A. Strobel, Montana State University, Personal communication). The reaction mixture minus D-alanine served briefly as a control prior to each determination in order to determine the blank oxygen consumption. The reaction was allowed to proceed for 5 to 30 minutes. With reference to the work of Boulanger and Osteux (1963) and Meister et al. (1960), the calculations of specific activity were based on the first few minutes of assay to better insure that the associated kinetics would approach zero order.

Enzyme Assay. Specific activity is expressed as milliunits (mU) of enzyme per milligram of protein. One milliunit is defined by the I.U.B.E.C. (1961) as that amount of enzyme which will catalyze the transformation of 1.0 millimicromole of substrate per minute. Thus, throughout this report, 1.0 mU of D-amino acid oxidase will refer to that amount of the enzyme which will catalyze the transformation

of 1.0 mμM of D-alanine per minute per milligram of protein at 25°C.

Induction of Enzyme Activity

D-Amino Acids. Germ-free Swiss mice (fifteen to thirty days old) received 1.0 mg per gram body weight of D-alanine (Nutritional Biochemicals Corporation, Cleveland, Ohio) on alternate days for a total of seven injections. All injections were administered through the intraperitoneal route. Additionally, germ-free C57B1/6K mice of comparable age were similarly treated with D-glutamic acid (Nutritional Biochemicals Corporation, Cleveland, Ohio). The sterile amino acid solutions were made up in pyrogen free PBS (Cutter Laboratories, Berkeley, California) and the pH was checked and regulated to 7.2 if necessary. Controls received comparable volumes of sterile pyrogen free PBS.

Heat Treated Bacillus cereus. In other experiments attempts were made to observe the induction of D-amino acid oxidase in GF Swiss mice which had received Bacillus cereus organisms treated according to a protocol for the preparation of heat stable somatic antigens (Campbell, 1964). The organisms were suspended in 0.3% formalin and PBS to a final concentration of 1×10^{10} organisms per ml. The sterile suspension was administered by gavage under the influence of

ether anesthesia using a 22 gauge flexible teflon needle (Hamilton Syringe Company, Los Angeles, California). The administration schedule was as follows:

Day	1	3	5	7	8	10	12
Amount (mls)	0.1	0.2	0.3	0.5	0.5	0.7	1.0

The animals were collected on the eighteenth day and the appropriate tissues from 16 animals were pooled and processed as described above. Untreated GF animals served as controls.

Cell Wall Preparations from Bacillus cereus. To induce D-amino acid oxidase activity in germ-free Swiss mice, a single dose of a cell wall preparation from Bacillus cereus which had been radiolabelled with ^{14}C-(U) D-alanine was given by gavage. The preparation of this material is described below.

Monocontamination. The possibility of the induction of D-amino acid oxidase in the tissues and organs of two sets of animals whose Trexler isolators had become monocontaminated with two different Bacillus spp. was checked. In the case of the mice monocontaminated with the organism designated Bacillus cereus which is described more fully below, the mice were monitored for enzyme activity and examined for monocontamination individually. Frequently, in cases of this type, not all animals in the unit were indeed monocontaminated; some in

fact, remained germ-free. This provided a useful opportunity for running unknown samples, in that the enzyme assays preceded the bacteriologic culture results by 24-48 hours. In no case, did a mouse showing no D-amino acid oxidase activity later prove to be contaminated or vice-versa. In the case of the unit monocontaminated with the organism designated Bacillus circulans, mice were randomized throughout the cages to insure uniform monocontamination. When multiple samples from each cage were indicative of monocontamination, all mice were removed from the unit, and peritoneal macrophages, kidneys, and liver were collected and pooled.

Tolerance. Attempts were made to induce a refractory state to D-amino acid oxidase induction analogous to immunologic tolerance. Newborn GF Swiss mice were to be given 1 mg per gram body weight of D-alanine in pyrogen free PBS pH 7.2 (Cutter Laboratories, Berkeley, California) every other day for 7 days. At 30 days of age a group was to be removed from the unit and examined for D-amino acid oxidase activity. If it was absent, attempts would be made to induce D-amino acid oxidase activity using cell wall preparations from Bacillus cereus. If no induction were to be observed, then attempts would be made to break this tolerance like state using standard immunological techniques.

Bacteriologic Methods

The two Bacillus spp. isolated from monocontaminated Trexler isolators were subjected to standard taxonomic procedures in an attempt to further characterize them.

Bacillus cereus. One was a Gram-positive rod, approximately 1-1.11 microns in diameter and having a length of 2 microns. Spores were central. The organism was motile by means of peritrichous flagella and grew equally well at 25°C and 37°C. Fermentation reactions showed that the organism produced no acid from mannitol with ammonium salts as the sole source of nitrogen and acid but no gas from glucose and sucrose. Litmus milk was peptonized and gelatin was rapidly liquefied. The organism grew in 7.5% NaCl broth but not on Simmon's citrate slants. Acetyl methyl carbinol was produced and nitrites were produced from nitrates. On these and other characteristics the organism most closely resembled Bacillus cereus.

Bacillus circulans. The other bacterium used in these studies was also a Gram-positive aerobe which had a length of 1.5-2.7 microns and a diameter of 0.7 microns. The spores were terminal to subterminal and swelled the cell to a diameter of 1.36 microns. No fermentation of mannitol with ammonium salts as a source of nitrogen

occurred nor did reduction of nitrate to nitrite. No change in the medium was observed when the organism was grown in litmus milk. Growth did not occur in the presence of 7.5% NaCl and gelatin was not liquefied. The organism was motile by means of peritrichous flagella. Acetyl methyl carbinol was not produced, nor was indole from tryptophan. Although the organism was tentatively identified as Bacillus circulans, this speciation could not be confirmed by Weaver and Smith (1969).

Radioisotopic Methods

Preparation of Labelled Bacillus cereus Cell Wall Material. Studies employing radioisotope methods were undertaken to determine the kinetics of D-amino acid oxidase induction following exposure of the synthetic apparatus of the host to bacterial cell wall extracts. Bacillus cereus cell wall materials were labelled to high specific activity by taking advantage of the fact that many Bacillus spp. can utilize ammonium salts or aliphatic amino acids as their sole source of nitrogen. A twenty-four hour culture of the Bacillus cereus strain previously described, growing in TSY broth, was inoculated into 1.0 liter of a modified basal salts medium. The basal salts medium contained: 0.5 g KH_2PO_4, 0.5 g K_2HPO_4, 0.2 g $MgSO_4$, and 10.0 g glucose to a final volume of 1000 ml in distilled

water. Additionally, one part per thousand of a trace element solution was added (Hutner, 1946). After incubation in this medium for one hour, a 5 ml aliquot was removed under aseptic conditions and was introduced into a sterile test tube. Fifty μc of D-alanine, uniformly labelled with ^{14}C, was suspended in 1.0 ml of PBS, and dispensed into the bacterial suspension using a 1.0 ml syringe and a sterile 13 mm Swinney filter holder containing a 0.45 micron Millipore filter (Millipore Corporation, Bedford, Massachusetts). Four additional 1 ml aliquots of PBS were administered via syringe to insure adequate washing of the labelled material from the filter. The bacterial suspension was incubated in the presence of labelled D-alanine for four days at room temperature. At the end of this period, the microorganisms were subjected to three passages through a French Pressure Cell at 15,000 pounds per square inch, or passage through a Raytheon Model OF 101 ultrasonicator for 15 to 20 minutes. Cellular lysis was verified microscopically. The cell wall fragments were collected by centrifugation at 35,000 rpm for 45 minutes and washed three times in PBS. The material was suspended in approximately 4.0 ml of PBS and sterilized by filtration through a 0.45 micron Millipore filter or by autoclaving for 15 minutes at a pressure of one atmosphere. Cell wall material prepared in this manner had a specific activity of approximately 80,000 disintegrations per minute (DPM)

per mg of dry weight.

The labelled cell wall material (approximately 4.44 mg) was introduced by gavage into ether on anesthetized GF mice. During a period of from 4 to 96 hours, treated and control mice were removed from the Trexler unit and weighed. Removal of blood from the tail was preceded by ether anesthesia. The collected blood was allowed to coagulate at room temperature and was placed in a $4^{\circ}C$ refrigerator overnight to allow for clot retraction. Following centrifugation at 1000 rpm for 15 minutes, the serum was removed with a capillary pipet and measured aliquots dispensed into a Spectravial II Nuclear Chicago scintillation vial. Tissues and organs were removed surgically, blotted on Whatman No. 1 filter paper and weighed. Small fragments were dissected away using irisectomy scissors. These fragments were weighed on a Mettler analytical balance and placed into a Spectravial II Nuclear Chicago scintillation vial. Following mechanical breakage with a small pestle, 2.0 ml of a 0.6N solution of NCS (Trademark of Nuclear Chicago Corporation) was added using a pipet. After digestion of the tissue for 24-48 hours, 17.5 ml of scintillation solvent was added using a Repipet (Lab Industries, Berkeley, California).

The solvent used in all cases consisted of 50 mg of 1,4-bis[2-(5-Phenyloxazolyl)] benzene and 8 grams of 2,5-Diphenyloxazole in 1000 ml of Toluene (Baker Analytical Reagents).

All samples were counted for 2 to 10 minutes in a Nuclear Chicago well type scintillation counter, and the values obtained corrected for internal quenching. Allowance was made for the weight of the sample and all values have been expressed as DPM per gram of tissue.

EXPERIMENTAL RESULTS

D-Amino Acid Oxidase Levels in Conventionally Reared and Germ-free Mice. There were relatively high levels of D-amino acid oxidase activity in the kidneys of 30- to 60-days old CR Swiss mice, whereas there was little or no enzyme activity in the kidneys of 30- to 60-days old GF Swiss mice. (Table I) Although the enzyme was detected in the kidney of one GF mouse, the specific activity of the enzyme preparation was markedly lower than that of any CR preparation. While the assay procedure did not incorporate flavine adenine dinucleotide (FAD), the addition of 17.6 μg of FAD to germ-free kidney preparations did not alter the results in later studies. Also, in later determinations, 17.6 μg of FAD was incorporated into the assay system on comparable CR animals, again with no alteration in the result. Boiled preparations of mouse kidney were inactive. When 50 μM of L-alanine was substituted for D-alanine in the assay system no activity resulted.

Since preliminary studies failed to demonstrate significant levels of D-amino acid oxidase activity in kidneys of GF mice in contrast to high levels in CR mice, it seemed reasonable to assume that substrates in the conventional environment permeated the bodies of CR mice and served to elevate enzyme levels. This assumption was supported by the observation that the sera of CR mice contained approximately 0.30 μmoles of D-alanine per ml, whereas the

Table I

D-Amino acid oxidase activity in kidney tissue of germ-free (GF), monocontaminated (MC), and conventionally reared (CR) mice

Strain	Status	No. showing activity	Specific activity[a]	Range[b]
Manor Swiss	CR	7/7	20.97	15.52-24.72
Manor Swiss	GF	1/14	0.31	0.00-4.31
Manor Swiss	MC	13/13	23.86	1.56-58.19
Manor Swiss	GF (D-alanine injected intraperitoneally	10/10	3.60	2.77-5.13
Manor Swiss	GF (phosphate-buffered saline injected intraperitoneally	0/9	0.07	0.0-0.37

[a] Mean mU of enzyme per mg of protein.

[b] Values less than 0.5 are not significant.

sera of GF mice contained no D-alanine (Hoeprich, 1965).

Induction of Enzyme Activity

Monocontamination. Since high levels of D-amino acids are commonly found in the cell walls of Gram-positive organisms (Martin, 1966) attempts were made to detect D-amino acid oxidase activity in the kidneys of formerly GF mice taken from a Trexler isolator which had been inadvertently monocontaminated with Bacillus cereus. In all cases fecal samples were obtained from the mice before they were removed from the unit and only data obtained from those mice demonstrating monocontamination (MC mice) with Bacillus cereus were included. Four of the mice taken from this unit were found to be germ-free and D-amino acid oxidase was not detectable in their kidneys. The data obtained (Table I) from these mice indicated that the Gram-positive organisms provided the inducing substrates which served to stimulate D-amino acid oxidase activity in the kidneys of GF mice to normal levels. The wide range in the data obtained from the MC mice probably reflects the variation in degree and time of exposure to inducing substrates provided by Bacillus cereus. Additionally, the kidneys and livers from a mouse population whose Trexler isolator had become monocontaminated with Bacillus circulans were pooled and assayed for D-amino acid oxidase activity. The

results obtained from this uniformly monocontaminated population indicated a specific activity of 7.03 mU of D-amino acid oxidase for the kidney pool whereas no activity was found to be associated with the liver pool. This would also indicate that Bacillus circulans is also able to provide substrates for the induction of the enzyme.

Heat Treated Bacillus cereus. In an attempt to differentiate between synthesis of D-amino acid oxidase on the part of the host and the possibility that the observed activity in CR and MC mice represented merely an accumulation of bacterial D-amino acid oxidase in the kidneys, GF mice received a heat inactivated sterile preparation of Bacillus cereus by gavage. That the D-amino acid oxidase activity observed does not simply represent an accumulation of bacterial D-amino acid oxidase was shown by the observation that a pool made up of the kidneys of 16 treated mice showed a specific activity of 5.07 mU compared to no activity in the kidney pool prepared from the kidneys of 7 GF controls.

D-Amino Acids. To further elucidate the nature of the inducing substrate and its role in the induction of the enzyme, enzyme assays were performed on kidneys of GF mice injected with a solution of D-alanine in pyrogen-free PBS. The results (Table I) indicate an active response of kidney tissue to contact with D-amino acids in the

environment. The differences in mean D-amino acid oxidase activities between the GF mice injected with D-alanine and their GF controls injected with pyrogen-free PBS are significant at the 95% level, as shown by a t test in which H_o: $(u_1=u_2/\sigma_1^2/\sigma_2^2)$ Bowker and Lieberman (1959). The D-amino acid oxidase levels of the GF mice injected with D-alanine were, however, significantly lower at the 95% level than those of the CR and MC groups (Bowker and Lieberman, 1959). Additionally, the efficacy of D-glutamate in the induction of D-amino acid oxidase was measured in preliminary experiments in the C57Bl/6K germ-free mouse. In this system, D-glutamic acid was, at best, as effective as D-alanine in inducing a D-amino acid oxidase which would deaminate D-glutamic acid. Unfortunately, the results were not expressed in terms of specific activity and were not repeated in the Swiss mouse. Therefore, no significance may be attached to them other than that they indicated that the induction of D-amino acid oxidase using D-glutamic acid should be attempted and would very probably be successful.

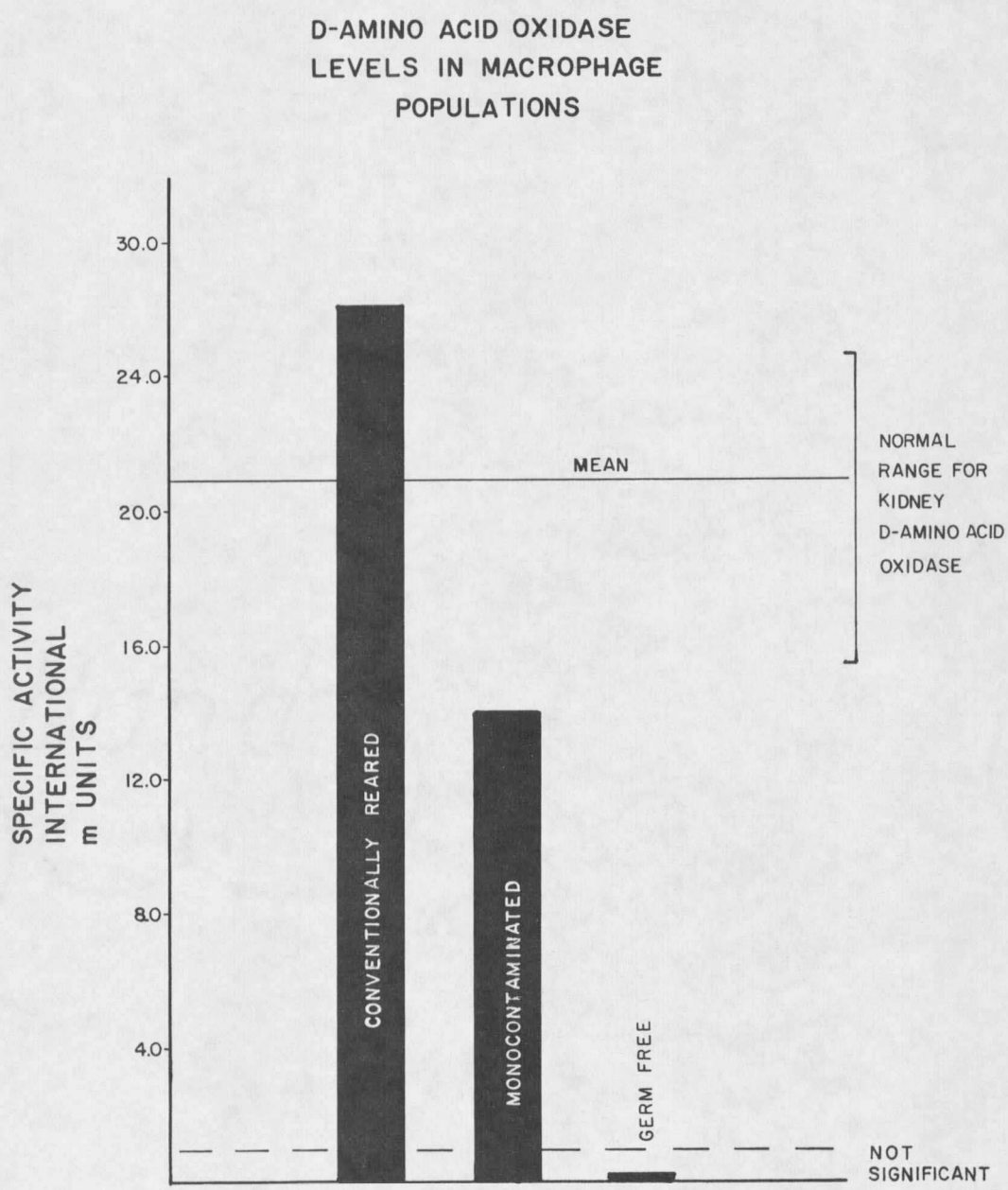
In Peritoneal Macrophages. The results of Cline and Lehrer (1969), in which D-amino acid oxidase was associated with the macrophages and polymorphonuclear leucocytes of humans and guinea pigs, raised the interesting question of whether D-amino acid oxidase was associated with the macrophages of the mouse. Hence, studies were

undertaken to determine whether a similar type of induction phenomena observed for the kidney was also applicable to the macrophage. Using macrophage populations derived from CR, GF and monocontaminated (Bacillus circulans) mice, and prepared as previously described, determinations of D-amino acid oxidase activity were made. The results are presented in Figure 1. As was the case in kidney tissue, CR mice demonstrated high levels of D-amino acid oxidase activity in their macrophages whereas macrophages derived from GF mice showed no activity. Macrophages collected from mice monocontaminated with Bacillus circulans showed significant levels of D-amino acid oxidase, however, the value did not approach the level obtained for CR mice. Interestingly enough, when the normal range of values for D-amino acid oxidase levels in CR mice (listed in Table I) is superimposed over Figure 1, it is readily apparent that the peritoneal macrophage of these mice is a somewhat richer source of D-amino acid oxidase than is the kidney. This phenomenon is also reflected in the MC (Bacillus circulans) group wherein a specific activity of 14.05 mU was shown for the macrophage in contrast to a value of 7.03 mU for the kidney.

Acquisition of Enzyme Activity

Normal Course in the Neonatal Conventionally Reared Mouse. As newborn CR mice, less than 24 hours old, had undetectable levels of

Figure I

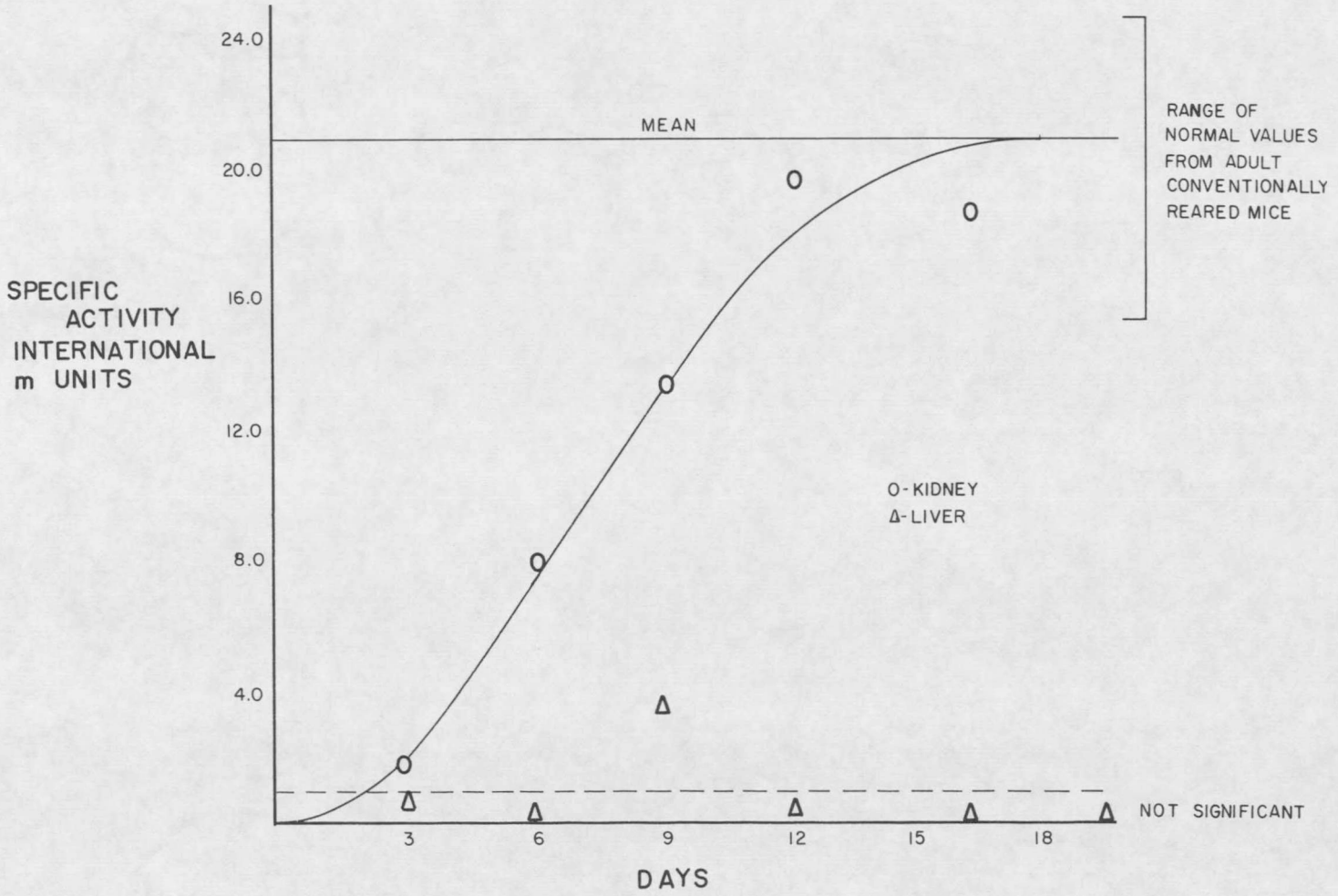

D-AMINO ACID OXIDASE
LEVELS IN MACROPHAGE
POPULATIONS
SPECIFIC ACTIVITY
INTERNATIONAL
m UNITS
30.0
24.0
20.0
16.0
12.0
8.0
4.0
MEAN
NORMAL
RANGE FOR
KIDNEY
D-AMINO ACID
OXIDASE
CONVENTIONALLY REARED
MONOCONTAMINATED
GERM FREE
NOT
SIGNIFICANT

D-amino acid oxidase, attempts were made to follow the normal course of acquisition of the enzyme during the first two weeks of life. The kidneys and livers of young CR mice were collected at 3, 6, 9, 12, and 15 days after birth. In all cases, the samples were collected from at least two litters for each time interval (a minimum of 20-30 individuals) and were pooled. Subsequent processing of these samples was according to the methods outlined above for the isolation of enzyme from kidney and liver tissues. The results (Figure 2) indicate that neonatal mice begin to acquire adult levels of D-amino acid oxidase by day 10 and that the process is essentially complete at 15-18 days. During the period of from approximately day 4 to 5 to day 9, the slope of the graph is linear with a value of 1.483. Therefore, during the period of maximal D-amino acid oxidase acquisition in the neonate enzyme activity is being acquired at the rate of approximately 1.5 mU per day. It is interesting to note in this regard that the neonatal mouse begins to acquire its normal microbial flora at approximately day 3 (Dubos, 1962). This would imply that there is a very direct relationship between the acquisition of the normal flora and the acquisition of D-amino acid oxidase.

The livers of these same neonatal mice were also examined for D-amino acid oxidase activity in a similar fashion. In confirmation of Shack's,(1943), findings, D-amino acid oxidase is apparently

Figure 2

The kinetics of D-amino acid oxidase aquisition in neonatal conventionally reared mice.



absent from the mouse liver. Although one pool of liver tissue (day 9) did manifest apparent D-amino acid oxidase activity, this was held to be an artifact and the reason for its occurrence remains unexplained.

Tolerance. Attempts to produce a tolerance like state with respect to D-amino acid oxidase synthesis met with no success. The injection of 1 mg per gram body weight of D-alanine in sterile pyrogen-free PBS by the intraperitoneal route into neonatal germ-free mice, less than 4 hours old, produced 100% deaths in all injected animals. Similar results were obtained when the initial injection was given at either 48 or 72 hours. When the dose was decreased to 0.1 mg per gram of body weight and the initial injection given at 72 hours, the mice would survive until the second injection given 48 hours later. Comparable volumes of pyrogen-free PBS injected into litter mate or non-litter mate controls produced no adverse affects. The mechanism of pathologic events leading to toxicity remains unresolved.

Radioisotopic Tracer Studies

Exposure Kinetics. In order to test the concept that substrates provided by the normal flora of the gastrointestinal tract crossed the barrier posed by the membranes of the intestinal tract and

exposed themselves to the synthetic mechanisms of the host, radio-labelled Bacillus cereus cell wall material was prepared and administered to GF mice. Although it has been shown previously, that antigenic moieties on the surface of bacterial cells expose themselves to the immune mechanisms of the host with the subsequent production of antibody, these results were not entirely amenable to extrapolation to the D-amino acid oxidase system. Therefore, these procedures were instituted in an attempt to determine the rapidity with which substrates on the cell surface would appear in the tissues and body fluids of the host. Additionally, it was of heuristic interest to determine the persistence of these compounds within the body and their relative rates of disappearance from various tissues, organs, and body fluids. The results of these experiments showing the disappearance of label from the serum, liver, kidney, and ileum are shown in Figures 3, 4, 5, and 6 respectively. In all cases, the ordinal values are expressed in terms of disintegrations per minute per gram of tissue with the exception of serum (Figure 3) in which they are expressed as DPM per ml. The figures represent the accumulation of three experiments and each point represents data from a minimum of 6 individuals. The horizontal bars represent one standard deviation to either side of the arithmetic mean. The data show that potential inducing substrates pass through the barrier

Figure 3

The loss of ^{14}C-(U) D-alanine labelled Bacillus cereus cell wall material from the serum of germ-free mice.

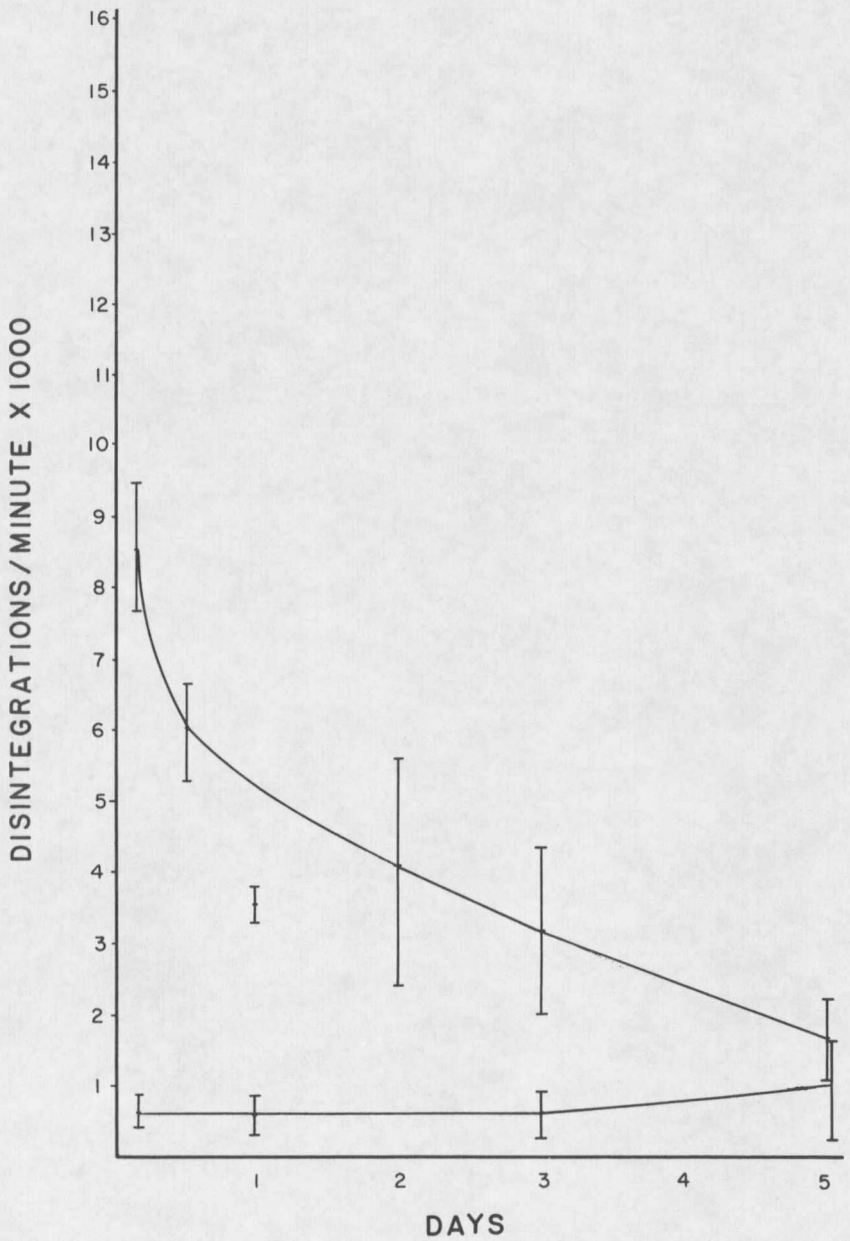


Figure 4

The loss of ^{14}C-(U) D-alanine labelled Bacillus cereus cell wall material from the livers of germ-free mice.

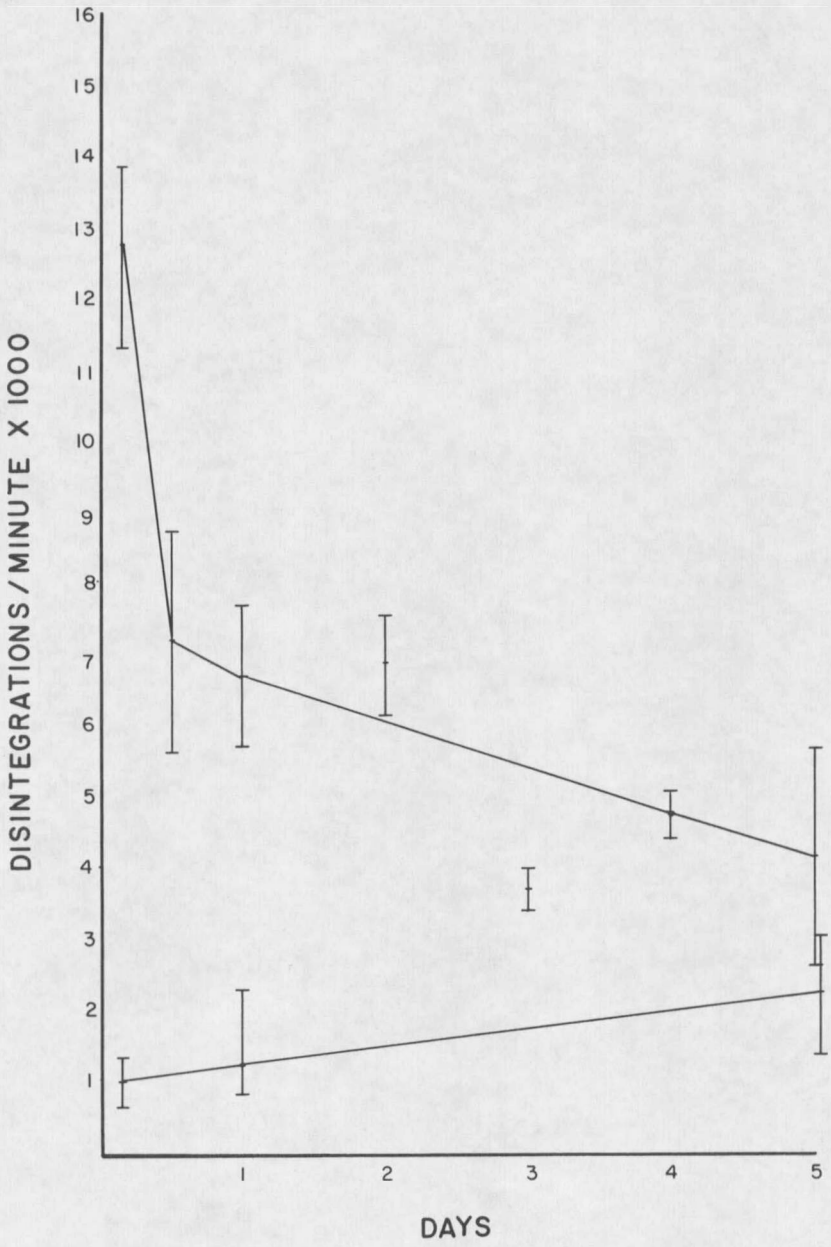


Figure 5

The loss of ^{14}C-(U) D-alanine labelled Bacillus cereus cell wall material from the kidneys of germ-free mice.

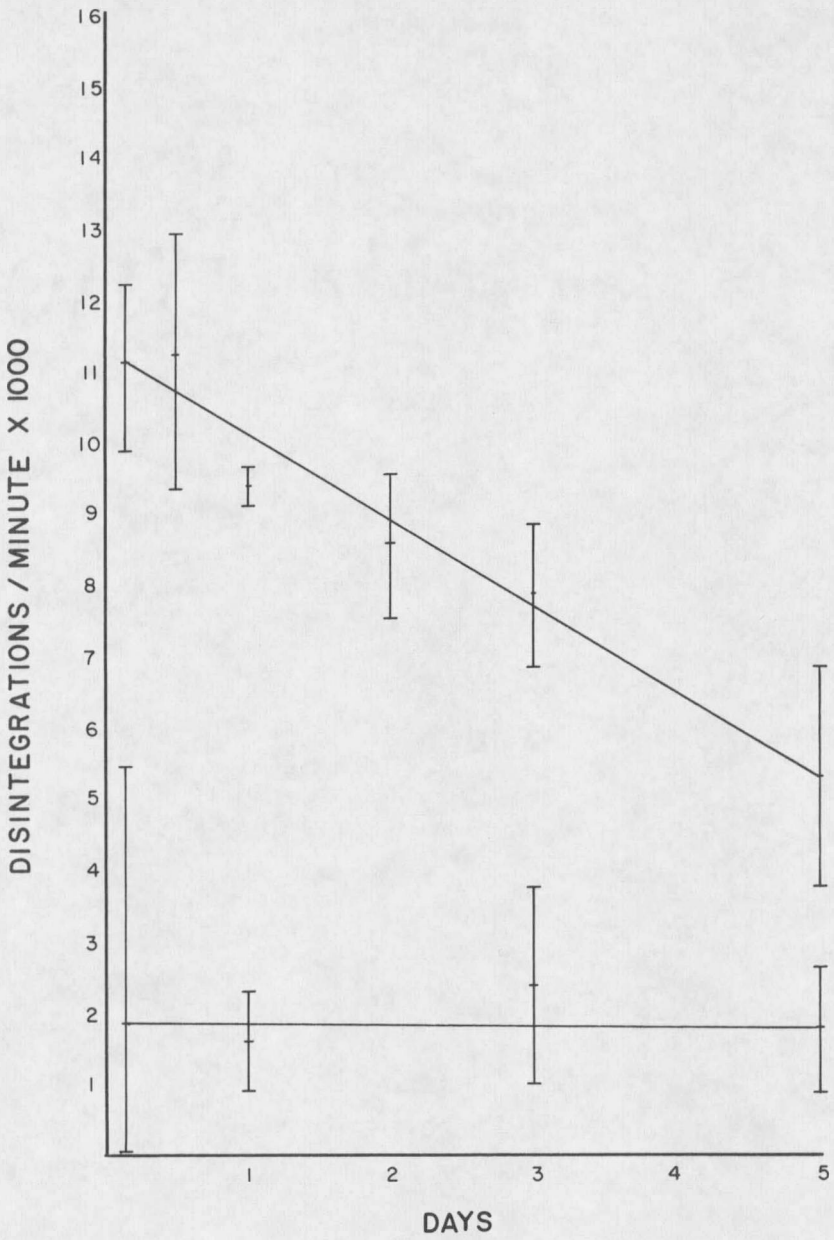
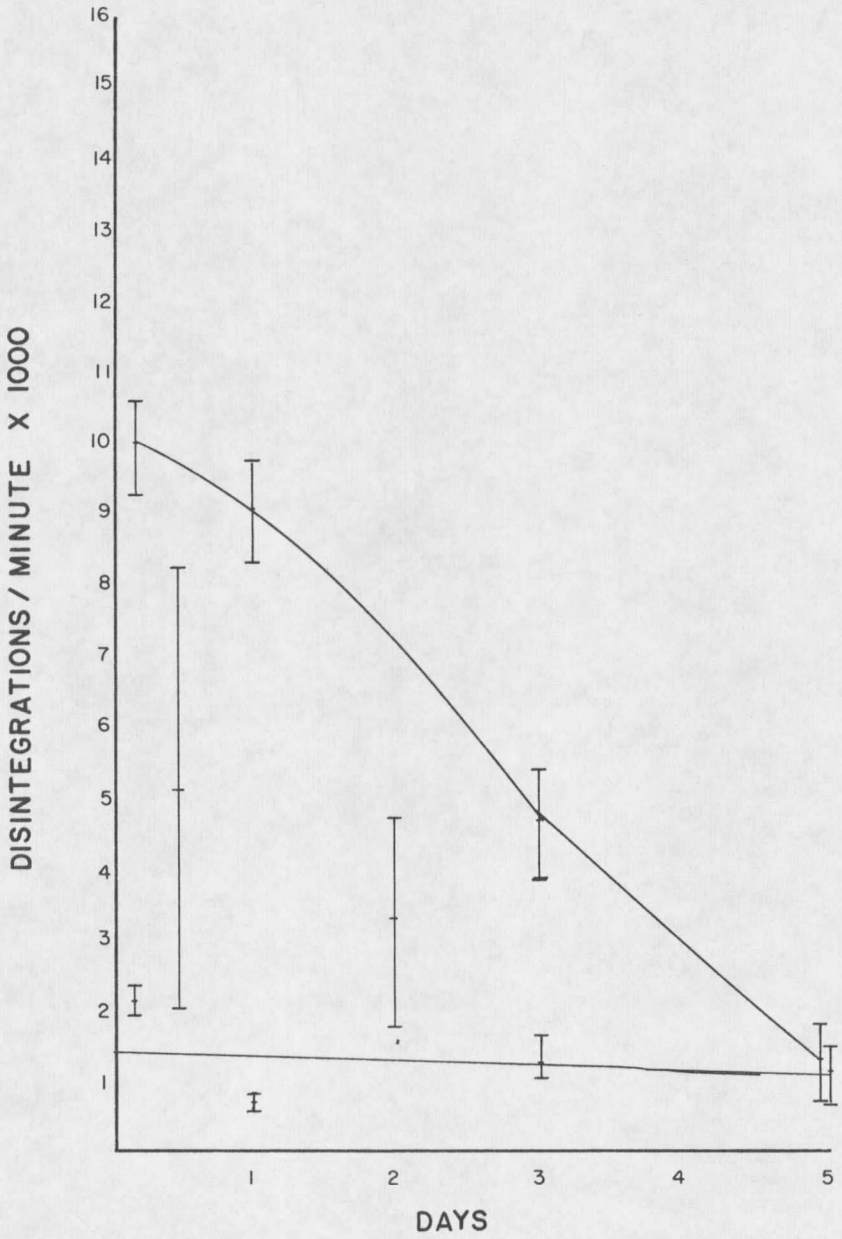


Figure 6

The loss of ^{14}C-(U) D-alanine labelled Bacillus cereus cell wall material from the ileums of germ-free mice.



imposed by the intestinal wall at a very rapid rate. In all cases, a significant degree of labelling is found in all tissues and fluids examined at 4 hours after gavage administration. In all cases labelling is maximal at 4 hours, with the exception of the kidney in which maximal labelling occurs at 12 hours.

The rate of decline in the amount of label is most rapid in the case of ileal tissue. This is consistent with the anatomical structure and physiological function of the organ. As the potential "storage capacity" of the organ is very limited, the passage of labelled materials from the lumen of the intestinal tract to the blood stream via the lacteals is apparently little delayed once the mucosal barrier is surmounted. The two aberrant points in Figure 6 are quite probably a reflection of the difficulty involved in preparing this material for scintillation counting. In addition to being refractory to the degradative action of NCS, the tissue is very tough, and difficult to disrupt by mechanical means. None of the points are a reflection of labelled material residing in adherent feces, for the lumen of the excised portion was thoroughly washed with PBS using a plastic wash bottle fitted with a capillary pipet as a nozzle.

The data obtained with respect to the serum (Figure 3) were consistent with the view that this fluid acts as a transport vehicle and is actually depositing most labelled materials in other tissues

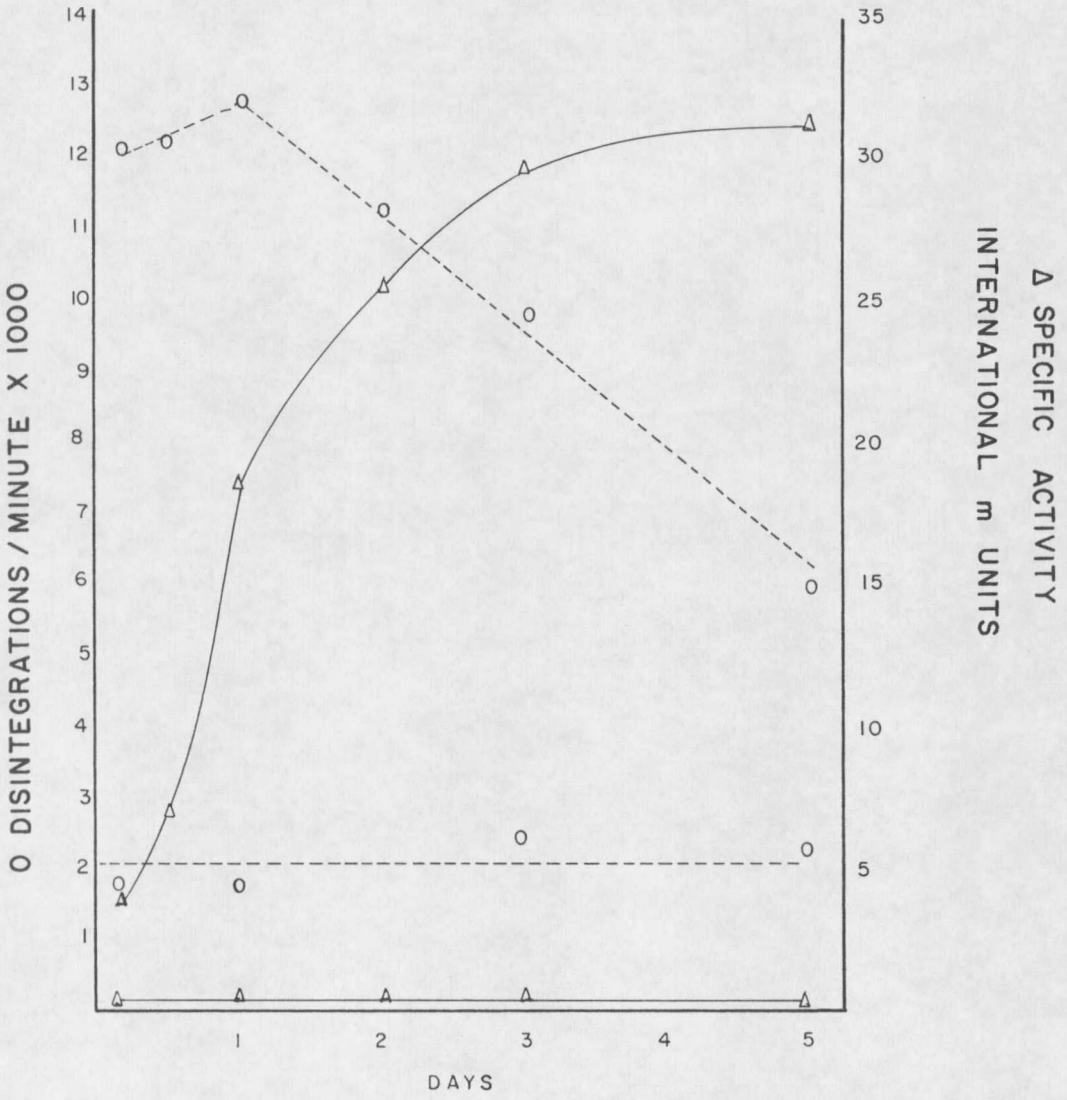
and organs. The relatively high degrees of labelling found in the liver (Figure 4) and the kidney (Figure 5) taken with the observation that they are the most lethargic with respect to elimination of the label, lead to the conclusion that they are the primary storage organs for the radiolabelled material. The data obtained for the liver (Figure 4) shows a biphasic curve, whereas the kidney (Figure 5) shows none of the rapid loss of labelled material between hours 4 and 12. The reasons for this are unknown, but one may speculate that if sufficient labelled substrate were able to expose itself very rapidly to the synthetic mechanisms of the host, then the production of D-amino acid oxidase might very rapidly ensue. If this were the case, then the high degree of labelling observed in the liver (Figure 4) at 4 hours might be substantially reduced at 12 hours if D-amino axid oxidase activity were to be accumulated during this time period by the kidneys and/or macrophages of the host. If this were to occur, the D-alanine on the bacterial cell wall would be deaminated to pyruvic acid, a material which is reabsorbed, and not excreted by the kidney. As the degree of labelling on the carbon atoms of D-alanine uniformly labelled with ^{14}C would not be affected by the deaminative action of D-amino acid oxidase, then uniformly labelled pyruvate would be returned to the liver to participate in a myriad of metabolic reactions. The kidney might indeed serve as a reservoir for

labelled material, a repository of D-amino acid oxidase activity, and a continuing source of uniformly labelled pyruvic acid to be disseminated throughout the body. A comparison of the slopes obtained for the liver (Figure 3) and kidney (Figure 4) during the period from 24 to 120 hours, shows them very roughly to be -26.01 DPM and -49.70 DPM per gram of tissue per hour, respectively. This is consistent with the view that the kidney may be a continuing source of labelled material, and may possibly at this time, contain D-amino acid oxidase activity.

Concomitant Radioisotopic Tracer Studies and D-Amino Acid Oxidase Induction. In an attempt to determine the merits, if any, of the above speculation, and to determine the induction kinetics for D-amino acid oxidase, an additional experiment was performed. Bacillus cereus cell wall material radiolabelled with ^{14}C-(U) D-alanine was prepared and administered to 60-day old GF mice as previously described. After small sections of kidney were removed for scintillation counting, the remaining portion of the two kidneys were pooled and assayed for D-amino acid oxidase activity. The amount of radioactivity and D-amino acid oxidase activity were followed simultaneously for each individual mouse. The results are shown in Figure 7. Each point represents the mean obtained from isotopic and enzymatic determinations on two animals that received radiolabelled cell wall

Figure 7

The concomitant loss of ^{14}C -(U) D-alanine labelled Bacillus cereus cell wall material and the induction of D-amino acid oxidase in the kidneys of germ-free mice.



material, and from one animal, for each time period, that served as an untreated control.

As is shown by the data, there is a reasonably direct relationship between the increase in D-amino acid oxidase activity and the disappearance of radiolabelled materials in the kidney. Furthermore, the appearance of radiolabelled material apparently serves to induce the synthesis of D-amino acid oxidase. Additionally, these data support the concept outlined in the preceding section; that the kidney may, at a very early time after the administration of label, contain significant levels of D-amino acid oxidase activity and may serve as a continuing source of labelled material which is then accumulated or retained in the liver.

Studies on Human Kidney Tissues

Specimens of human kidney material were obtained through the cooperation of Dr. V.W. Steele, Pathologist, Bozeman Deaconess Hospital.

Cortical tissue was removed from the kidney sample and plunged immediately into the cold sucrose solution previously described. As soon as possible thereafter, the samples were frozen and were stored in the frozen state at -20°C until analysis. A specimen obtained from a 5 month old fetus showed no D-amino acid oxidase activity. In

contrast, a hydronephrotic specimen removed from a 43 year old female patient showed a specific activity of 13.70 mU of D-amino acid oxidase activity. Nothing in these results indicate that humans do not also have D-amino acid oxidase activity in their kidneys, and probably acquire it shortly after birth. However, any conclusions about the distribution, acquisition, concentration, and inducibility of human D-amino acid oxidase must await the results of systematic studies.

DISCUSSION

On the basis of these observations it is concluded that D-amino acid oxidase in the Swiss mouse is synthesized in response to D-amino acids, presumably derived from the degradation of cell wall components of Gram-positive organisms which are part of the normal flora of the gastrointestinal tract. That induction of the enzyme does not simply represent an accumulation of bacterial D-amino acid oxidase in the kidneys was illustrated by a marked increase in enzyme activity in GF mice injected with the D-amino acids, D-alanine and D-glutamic acid. Additional support for this assertion is provided by the observations that the kidneys of mice who received sterile heat treated preparations of Bacillus cereus showed high degrees of D-amino acid oxidase activity. Furthermore, cell wall material from Bacillus cereus organisms was wholly efficacious in stimulating the rapid synthesis of D-amino acid oxidase to fully adult levels.

These results do not entirely support those of Meister et al. (1961), who found equal concentrations of D-amino acid oxidase in the kidneys of CR and GF mice. As previously mentioned (Lyle and Jutila, 1968) this discrepancy might be related to (i) differences in the enzyme assay system, (ii) differences in the nutrition of the GF animals, (iii) differences in the mouse strains employed in the study, or to a combination of the three. The observation that 30- to 60-day old GF mice possess little or no D-amino acid oxidase was somewhat

unexpected. However, when one considers that Purina Laboratory Chow #5010 C contains less than 1000 microbial organisms per gram, and that not all may provide D-amino acids, then the maximal exposure to potential inducing substrates that a 60-day old GF mouse would receive is relatively low. Assuming the mouse to be weaned at day 21 and that it subsequently ingests an average of 1.0 gram of feed per day, than the maximal possible accumulative exposure to bacterial carcasses during its 60 day life span would be 39,000 organisms. Presumably, none would be passively received through the lacteal secretions of the mother, for Hoeprich (1965) has shown that the serum of the GF guinea pig contains no D-alanine, and an analogous situation probably exists in the mouse. In contrast, those mice that received, by gavage, sterile heat treated preparations of Bacillus cereus were exposed to the 39,000 bacterial carcasses acquired through the feed and approximately 3.3×10^{10} bacterial carcasses as a result of the administration protocol. These mice registered a kidney D-amino acid oxidase specific activity of 5.07 mU compared to no activity in the kidneys of their GF controls. However, the differences in degree of potential exposure to inducing substrates approximate six orders of magnitude. In any case, the level of D-amino acids contained in dead bacterial bodies present in the feed of the GF mouse appeared, throughout these experiments, to be

insufficient for the stimulation of detectable levels of the enzyme. It was only when additional substrate was provided in the form of D-amino acids, monocontamination with live Bacillus cereus or Bacillus circulans, heat treated Bacillus cereus, cell wall preparations derived from Bacillus cereus, or the conventional contaminated environment, that the accumulation of D-amino acid oxidase was observed. It very well may be that the induction of D-amino acid oxidase is a quantitative phenomenon, and associated with it is a threshold effect. If this is so, why then did the D-alanine injected mice, who received far and away the greatest exposure to potentially inducing substrates, show so little D-amino acid oxidase activity? This situation may be analogous to that found in D-amino acid oxidase inducible systems in bacteria; in these systems, it has been found that D-amino acids are not as efficacious as polypeptides containing D-amino acids for the induction of the enzyme (Dr. H.A. Barker, University of California, Personal communication).

A similar situation may have been encountered here, in that the data suggest that live proliferative bacterial populations supplied by monocontamination, heat-treated Bacillus cereus or cell wall material derived from Bacillus cereus are all more effective inducers of the enzyme than is D-alanine. It should also be kept in mind, that Van Bekkum and Nieuwerkerk (1965) were entirely successful in

producing a state analogous to immunologic tolerance with respect to the synthesis of tryptophan pyrrolase. To be sure, this was accomplished in the newborn and not in animals 15 days of age. However, Gill, (1965), has shown that some very dramatic differences exist in the ability of the immunocompetent rabbit to respond to synthetic antigens composed of L-amino acids or on the other hand, an analogous antigen composed of D-amino acids. A given dose of antigen composed solely of L-amino acids that elicited the copious production of antibody, resulted in the production of immunologic paralysis when the same dosage of analogous antigen composed of D-amino acids was administered. It was only through the reduction in relative immunizing dosage, that the production of antibody to antigens composed of D-amino acids was accomplished. These considerations led to attempts to induce the counterpart of neonatal immunologic tolerance for D-amino acid oxidase that has already been observed in the case of tryptophan pyrrolase by Van Bekkum and Nieuwerkerk (1965). Unfortunately, these preliminary efforts to accomplish this in vivo met with no success. The results of these experiments do suggest an alternative, however. No D-amino acid oxidase activity was observed in peritoneal macrophages derived from germ-free mice, whereas it was found in macrophages derived from CR and mice monocontaminated with Bacillus circulans. Therefore, attempts to produce the analogue of

immunologic tolerance in an *in vitro* macrophage culture system might very well meet with success.

The observation made in these experiments, that the macrophages of CR mice and those from mice monocontaminated with *Bacillus circulans*, possess relatively higher levels of D-amino acid oxidase than do their kidneys, was not observed in the case of the guinea pig by Cline and Lehrer, (1969). Perhaps the mouse is unique in this respect as it appears to be with respect to the absence of D-amino acid oxidase activity in the liver (Shack, 1943) and (Meister *et al.*, 1961). An additional consideration may be that our mouse colony is unique in that Cantrell and Jutila (In press), have consistently found that *Bacillus spp.* contribute significantly to the normal flora of our CR mice. Monocontamination of mice with any *Bacillus spp.* would of course, introduce an enormous degree of abnormality into the "induction pressure" characteristically provided by the normal gastrointestinal flora. It is also interesting to note, with respect to the work of Cline and Lehrer (1969), that they carried out their determinations at 37°C whereas these determinations were made at 25°C. While this should not alter the basic relationships between the amount of D-amino acid oxidase activity in the macrophage and kidney, the activity they report may not be truly indicative of the actual dynamics. While the D-alanine concentration is entirely adequate to

insure zero order kinetics, the rate of the reaction may also be limited by the availability of oxygen. Above 30°C there is very little oxygen remaining in solution, and of course at 37°C, there is even less (Dr. G.A. Strobel, Montana State University, Personal communication).

The results obtained from studies in which radiolabelled Bacillus cereus cell wall material was administered by gavage to GF mice indicate that the barrier imposed by the gastrointestinal mucosea is not insurmountable. Minute breeches in the integrity of this tissue undoubtedly occur as the result of abrasion by food materials during peristalsis normal debridement and tissue replacement, and the action of bacterial mucinases, hyaluronidases or other necrotic factors. Under these circumstances, the rapidity with which potential inducing substrates or antigenic moieties may reach their appropriate target tissues is startling, but not surprising.

The very rapid increase in kidney D-amino acid oxidase levels and the concomitant decrease in the amount of ^{14}C radiolabelling associated with that organ, are directly suggestive of an inducible nature of D-amino acid oxidase in the GF Swiss mouse. This rapidity of synthesis and apparent substrate (D-alanine) induction are furthermore suggestive of a classical inducible enzyme in the sense of the β-galactosidase model, Jacob and Monod (1961). Product (pyruvic acid)

repression was not directly observed. However, the ultimate leveling of specific activity observed at days 12-15 in the course of acquisition in neonatal mice, and that observed at 3 to 5 days in the concomitant isotopic-induction experiment, are suggestive of negative feedback control. It is not suggested however, that mice behave in a fashion similar to bacteria with respect to their inducible enzymes. Van Bekkum and Nieuwerkerk (1965) have clearly shown that the substrate inducible tryptophan pyrrolase system is susceptible to a situation analogous to immunological tolerance. As the normal course of acquisition of D-amino acid oxidase by the neonatal mouse is almost identical to the acquisition of tryptophan pyrrolase in the rat, it would be of interest to learn whether a similar situation exists with respect to this enzyme. If this were so, D-amino acid oxidase might very well represent a primitive immune mechanism. The confirmation or refutation of this suggestion will, however, have to await the solution of dosage problems in the neonatal GF mouse, the development of a successful in vitro macrophage culture system, or attempts to suppress the normal course of acquisition of the enzyme in the neonatal CR mouse. As the dosage levels of D-alanine which produced 100% deaths in newborn GF mice are harmless in a 15 to 30 day old GF mouse, one may speculate that the deleterious effects observed are, to some degree, dependent upon the relative incompetence

of the mouse at this age to accomplish protein synthesis. It is known for example, that neonatal mice are not fully immunocompetent until 72-120 hours after birth (Reed and Jutila, 1967). Therefore, one may postulate that D-alanine competively inhibits an acetyl t-RNA transferase when given in high dosages to neonatal mice.

Although the experiments presented in this report have been primarily concerned with the induction of D-amino acid oxidase with D-alanine, nothing is presented to imply that other D-amino acids would not be as efficacious if not more so in accomplishing induction. Additionally, no considerations are present which would rule out possible nonspecific induction factors. The results of these experiments do however, strongly suggest that D-amino acid oxidase is an inducible enzyme in the GF Swiss mouse, and that the normal bacterial flora of the gastrointestinal tract may play an important role in the induction of host enzymes. This concept has also been proposed by Van Bekkum and Nieuwerkerk (1965) in a study concerning the induction of tryptophan pyrrolase.

SUMMARY

High levels of D-amino acid oxidase activity were found in the kidneys of conventionally reared mice, whereas the enzyme was absent from the kidneys of most germ-free mice. Injection of D-alanine, monocontamination with Bacillus cereus or Bacillus circulans, administration of heat killed Bacillus cereus or cell wall preparations from this organism, stimulated D-amino acid oxidase activity in the kidneys of germ-free mice. Some of these same effects were also observed in the macrophages of germ-free mice. Acquisition of the enzyme occurred progressively for a week after birth of conventionally reared mice and adult levels were found in all mice after 10 days of age.

On the basis of these observations, it is concluded that D-amino acid oxidase in the Swiss mouse is synthesized in response to D-amino acids, presumably derived from the degradation of cell wall components of gram-positive organisms which are part of the normal flora of the gastrointestinal tract.

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