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(54) **PROCEDE POUR IDENTIFIER LE SITE D'ACTION D'AGENTS
CHIMIQUES XENOBIOTIQUES**

(54) **A METHOD FOR IDENTIFYING THE SITE OF ACTION OF
XENOBIOTIC CHEMICALS**

(57) Cette invention se rapporte à un procédé permettant de découvrir le site d'action de composés xénobiotiques. Ce procédé consiste à soumettre une cellule détecteur comprenant un promoteur sensible aux agents génotoxiques liés opérationnellement à un complexe de gènes reporteurs luminescents. L'exposition des bactéries détecteurs à des composés qui sont génotoxiques produit un accroissement de la luminescence. Ces composés sont groupés et peuvent être analysés pour que leur activité pharmaceutique soit déterminée. L'exposition des bactéries détecteurs à des agents xénobiotiques actifs qui ne sont pas génotoxiques produit une inhibition de la bioluminescence ou de la luminescence stable. En soumettant les bactéries détecteurs à un tri, tel qu'un titrage génétique ou d'inversion nutritionnelle, on dévoile le site d'action du composé en question.

(57) A method is disclosed for the discovery of the site of action of xenobiotic compounds. The method involves subjecting a detector cell comprising a genotoxic-sensitive promoter operably linked to a luminescent reporter gene complex. Exposure of the detector bacteria to compounds that are genotoxic results in an increase in luminescence. Such compounds are grouped and may be analyzed for pharmaceutical activity. Exposure of the detector bacteria to active xenobiotics that are not genotoxic results in inhibition of bioluminescence or stable luminescence. Subjection of the detector bacteria to a screen such as nutritional reversal or genetic titration reveals the site of action of the compound.

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(21) International Application Number: PCT/US98/03684 (22) International Filing Date: 26 February 1998 (26.02.98) (30) Priority Data: 60/039,582 28 February 1997 (28.02.97) US (71) Applicant (for all designated States except US): E.I. DU PONT DE NEMOURS AND COMPANY [US/US]; 1007 Market Street, Wilmington, DE 19898 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): ELSEMORE, David, Allen [US/US]; 1223 Town Court North, Lawrenceville, NJ 08648 (US). LAROSSA, Robert, Alan [US/US]; 672 Kadar Drive, West Chester, PA 19382 (US). SMULSKI, Dana, Robin [US/US]; 603 Greenbank Road, Wilmington, DE 19808 (US). VAN DYK, Tina, Kangas [-/US]; 119 Cambridge Drive, Wilmington, DE 19803 (US). (74) Agents: FLOYD, Linda, A. et al.; E.I. du Pont de Nemours and Company, Legal Patent Records Center, 1007 Market Street, Wilmington, DE 19898 (US).		(81) Designated States: AL, AM, AU, AZ, BA, BB, BG, BR, BY, CA, CN, CU, CZ, EE, GE, GW, HU, ID, IL, IS, JP, KG, KP, KR, KZ, LC, LK, LR, LT, LV, MD, MG, MK, MN, MX, NO, NZ, PL, RO, RU, SG, SI, SK, SL, TJ, TM, TR, TT, UA, US, UZ, VN, YU, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>With an indication in relation to a deposited microorganism furnished under Rule 13^{bis} separately from the description.</i> <i>Date of receipt by the International Bureau:</i> 26 February 1998 (26.02.98) Date of publication of the amended claims: 5 November 1998 (05.11.98)
(54) Title: A METHOD FOR IDENTIFYING THE SITE OF ACTION OF XENOBIOTIC CHEMICALS (57) Abstract A method is disclosed for the discovery of the site of action of xenobiotic compounds. The method involves subjecting a detector cell comprising a genotoxic-sensitive promoter operably linked to a luminescent reporter gene complex. Exposure of the detector bacteria to compounds that are genotoxic results in an increase in luminescence. Such compounds are grouped and may be analyzed for pharmaceutical activity. Exposure of the detector bacteria to active xenobiotics that are not genotoxic results in inhibition of bioluminescence or stable luminescence. Subjection of the detector bacteria to a screen such as nutritional reversal or genetic titration reveals the site of action of the compound.		

TITLEA METHOD FOR IDENTIFYING THE SITE OF
ACTION OF XENOBIOTIC CHEMICALSFIELD OF INVENTION

5 The invention relates to field of molecular biology and to methods for screening compounds for biological activity. More specifically, a method has been developed to rapidly identify the site of action of various xenobiotic compounds in particular, xenobiotic agrochemicals and antimicrobials.

BACKGROUND OF THE INVENTION

10 Technological advances within the chemical arts have made possible the synthesis of vast arrays of chemical compounds of interest to the agrochemical, pharmaceutical and environmental industries. These methods of synthesis are capable of producing far more compounds than can be reasonably screened to identify their utility. The utility of these compounds is modeled on various
15 structure-function relationships and is often confirmed through screening methods that associate these compounds with desired, known activities. Once a compound or class of compounds has been subjected to a screen and a putative activity has been determined, further analysis is needed to determine the mechanism also known as site of action, i.e., the target, of the compound. The effort to understand
20 the precise site of action of a chemical or environmental agent is driven by a concern for environmental safety and a desire to capture the value associated with this understanding for use in the agrochemical and pharmaceutical industries. A need exists, therefore, for methods of determining the site of action of synthetic xenobiotic compounds that are rapid, accurate and able to accommodate the vast
25 numbers of compounds being produced.

 Methods for determining the site of action of various compounds have been developed using the complementary methods of nutritional reversal and genetic titration in microbially based systems. LaRossa et al., [*J Biol. Chem.*, 259 (14), (1984) 8753-8757] teach a pool strategy for nutritional reversal previously
30 used to classify the pathway effected by an auxotrophic mutation, termed auxanography, [Davis, R. W., D. Botstein and J. R. Roth: A Manual For Genetic Engineering: Advanced Bacterial Genetics. X+254p. Cold Spring Harbor Laboratory: Cold Spring Harbor, N.Y., U.S.A., 1980]. This method precisely defines the site of sulfonylurea herbicide action in *Salmonella typhimurium* LT2.
35 The method uses lawns of bacterial cells grown on solid media and exposed to effective concentrations of herbicide. Sulfonylurea herbicides inhibit *Salmonella* growth by inhibiting the branched chain amino acid biosynthetic pathway. Nutrient supplementation overcome specific amino acid deficiencies imposed by herbicidal action. Results are seen in about 48 hr. The method of LaRossa et al.

is useful for determining a site of action for a specific active xenobiotic compound but, like whole plant assays, is time consuming and requires large amounts of test compound to be screened.

Genetic titration in a microbial population offers an alternative to nutritional reversal. Genetic titration has been used during the past 40 years. Selection of regulatory mutations that increase the titer of pathway enzymes in response to a challenge with an antimetabolite are well known in the *E. coli* and *S. typhimurium* literature. Gene duplications have been selected in both mammalian cell lines (using drugs and transition state analogs) and with antimetabolites in bacteria. These selections are based upon the increased enzyme content of the cell titering out the deleterious effect of the inhibitor. Systematic selection for such titrations using antimetabolites in yeast in conjunction with multicopy recombinant DNA based yeast chromosomal libraries were pioneered by Falco [Falco et al., *Genetics* 109:21-35 (1985)] and Rine [Rine et al., *Proc Natl Acad Sci U S A* 80: 6750-4 (1983)]. More recently, Sternberg and Chattajee [PNAS 92:8950-8954 (1995)] have used this recombinant DNA based methodology in *E. coli*. This art teaches the use of the method of genetic titration in yeast to identify various macromolecular targets of a crop protection chemical and a few pharmaceuticals. It also teaches that recombinant DNA mediated genetic titration may identify a bacterial target.

Genetic titration proceeds generally by first preparing DNA libraries of a bacterial genome in multicopy cloning vectors where each portion of the genome is highly amplified. Bacterial hosts are transformed with the library and screened for growth on plates containing effective inhibitory concentrations of the xenobiotic compound to be tested. Colonies are picked and the plasmid isolated and sequenced. The sequence is compared to known sequences to identify genes that could encode a site of action for the compound.

These methods of nutritional reversal and genetic titration are both limited by the need to place cells of bacteria or yeast on agar plates and subsequently score or monitor for cell growth in response to the presence of the compound. This limitation makes the methods slow and cumbersome. Further, neither of these methods offers a way to determine the relative physiological or environmental toxicity of the compounds being screened. One of the significant disadvantages of any current screening method is that many active compounds may be identified in initial screens that must be later discarded due to poor results in toxicological screens. Incorporation of a toxicological pre-screen into the site of action screen would minimize this costly investment of time and resources.

Bioluminescent reporters are known as toxicological detectors in the art. One of the most common are genes encoding the firefly luciferase. Another is a

set of five genes, *luxCDABE*, that has been isolated from the bioluminescent bacteria *Vibrio fischeri*. Both eukaryotic and prokaryotic genes have been used in recombinant systems to serve as detectors.

5 cDNA encoding firefly luciferase has been expressed in *E. coli* under the control of the *lacZ* promoter [Tatsumi et al., *Biochem. Biophys Acta.*, 1131, (2), pp 161-165, (1992)], and the *luxAB* fusion gene has been expressed in *Bacillus* at levels comparable to those achievable in *E. coli* by placing it under the control of the powerful *P_{xyn}* promoter (Jacobs et al., *Mol. Gen. Genet.*, 230(1-2), pp 251-256, (1991)).

10 Bacterial genes have also been isolated and expressed. Rychert et al., [*Environ. Toxic. Water Qual.*, 6 (4), pp 415-422, (1991)] have shown that recombinant *E. coli* harboring the plasmid, pJE202, that contains the promoterless *Vibrio fischeri lux* gene complex, was sensitive to Zn^{2+} , ethidium bromide, sodium pentachlorophosphate, Cu^{2+} and 2,4-dichlorophenoxyacetic acid. Response in
15 this assay was registered by a decrease in baseline light emitted by the transformed *E. coli*.

In addition to the use of the promoterless bacterial gene complex, foreign promoters have been fused to the bacterial bioluminescent structural genes for use in assays specific to various toxicants. For example, recombinant bacteria have
20 been developed by fusing the *lux* gene complex to chemically responsive bacterial promoters and then placing such chimeras in appropriate hosts. These recombinant bacteria are sensor organisms that glow in response to specific stimuli. An example of this type of gene fusion is described by Burlage et al., [*J. Bacteriol.*, 172 (9) pp 4749-4757 (1990)] where a DNA fragment from plasmid
25 NAH7 containing a promoter for the naphthalene degradation pathway was fused to the *lux* genes of *Vibrio fischeri* and used to transform a strain of *Pseudomonas*. The resulting transformant displayed an increase in light emission in the presence of naphthalene. The induction of bioluminescence was demonstrated to coincide with naphthalene degradation by the transformed organism.

30 Another test system specifically responsive to mercury (Hg) is described by H. Molders (EP456667). Here, indicator bacterial strains are provided (by vector-mediated gene transfer) containing a *mer* promoter, specifically inducible by Hg ions, fused to a bacterial luciferase (*lux AB*) genes complex which is responsible for bioluminescence. The test system of Molders relies on the
35 induction of the *mer* promoter by the presence of mercury and the subsequent increase in light emission from the recombinant bacteria.

In contrast to test systems specifically-responsive to chemicals, a prokaryotic system that measures a global regulatory response to a variety of chemicals is described by Van Dyk et al. [*Appl. Environ. Microbiol.* 60 (5),

pp. 1414-1420 (1994)]. In this system, *E. coli* strains containing fusions of heat shock promoters from *E. coli* to the *lux* genes of *Vibrio fischeri* responded by increased bioluminescence to many chemical stresses.

Measurement of eukaryotic stress responses as a method to determine chemical toxicity was described by Farr and Todd (WO 94 17208). Here, the disclosure provided a method to measure transcription and translation levels from genes linked to native eukaryotic stress promoters. At least one stress promoter from each of the following groups is utilized: redox stress, DNA stress, protein stress and energy/ionic stress. This method yields information on the mode of action of the chemical in eukaryotic cells; however, such information will not directly yield information on the site of action of the chemical.

Applicants have previously disclosed the use of various prokaryotic stress promoters, including those sensitive to genotoxic stress, in commonly owned U.S. Patent No. 5,731,153, issued 24 March 1997 and U.S. Patent No. 5,683,868, issued 4 November '997 (WO 94 13831). WO 94 13831 teaches the use of detector organisms containing a stress promoter-bioluminescent gene fusion to detect various environmental stresses including those sensitive to protein damage (heat shock), DNA damage (genotoxic), oxidative damage, cell membrane damage, amino acid starvation, carbon starvation, and nitrogen starvation. U.S. Patent No. 5,731,163 demonstrates the use of similarly transformed detector cells as lyophilized reagents. Finally, in commonly owned USSN 08/735,545 Applicants teach the use of bioluminescent reporters fused to bacterial promoters isolated from restriction of genomic DNA for the identification of new, compound sensitive promoters.

The problem to be overcome therefore is to develop a rapid, facile method for the identification of the site of action of agrochemically or pharmaceutically active xenobiotic compounds that are non-toxic to animals, including humans, or the environment.

30 SUMMARY OF THE INVENTION

The present invention provides a method for the identification of the site of action of a stressor comprising:

(i) contacting a stressor with a detector bacteria, the detector bacteria comprising a genotoxic-sensitive promoter operably linked to a luminescent reporter gene complex to form a gene fusion that confers a bioluminescent positive phenotype upon the detector bacteria wherein exposure of the genotoxic-sensitive promoter to genotoxic compounds drives heightened expression of the luminescent reporter gene complex producing an increased bioluminescent signal;

(ii) selecting for genotoxic or non-genotoxic stressors capable of inhibiting the growth of the detector bacteria of step (i) by monitoring the growth and light output of the detector bacteria;

(iii) submitting the growth-inhibiting, genotoxic or non-genotoxic stressor selected in step (ii) to a site of action screen;

(iv) identifying the site of action in the detector bacteria; and

(v) confirming the site of action in a target organism.

The present invention further provides detector bacteria strains comprising a *recA-LuxCDABE* gene fusion as well as non-bioluminescent parent strains possessing a multiplicity of cellular and membrane mutations.

The invention further provides methods for determining whether a compound is genotoxic and comprising the steps of:

(i) culturing a detector cell comprising a promoter regulated by a SOS bacterial regulatory circuit and a *luxCDABE* gene complex wherein the *luxCDABE* gene complex is positioned in the bacterial chromosome downstream of the SOS promoter such that when the SOS promoter is expressed, then the *luxCDABE* gene complex is also expressed;

(ii) contacting the culture with a substance to be tested; and

(iii) determining whether the substance is genotoxic by measuring the amount of luminescence in the culture.

The invention additionally provides methods of identifying a structural gene encoding a stressor target comprising:

(i) contacting a stressor with a detector bacteria, the bacteria comprising a genotoxic-sensitive promoter operably linked to a luminescent reporter gene complex to form a gene fusion that confers a bioluminescent positive phenotype upon the detector bacteria wherein exposure of the genotoxic-sensitive promoter to genotoxic compounds drives heightened expression of the luminescent reporter gene complex producing an increased bioluminescent signal;

(ii) selecting for genotoxic or non-genotoxic stressors capable of inhibiting the growth the detector bacteria of step (i) by monitoring the growth and light output of the detector bacteria;

(iii) submitting the growth-inhibiting, genotoxic or non-genotoxic stressor to a site of action screen wherein the stressor target is identified and;

(iv) isolating the structural gene encoding the stressor target.

In another embodiment, the invention provides methods for identifying compounds having glyphosate-like activity, thienylalanine-like activity and ALS-inhibitory activity.

BRIEF DESCRIPTION OF THE FIGURES, BIOLOGICAL
DEPOSITS AND SEQUENCE LISTING

Figure 1 (flow chart-strains 1a and 1b) is a diagram showing the genealogies of the detector cells used in the present invention. Solid arrows indicate construct of non-bioluminescent strains. Broken arrows indicate construction of bioluminescent derivatives. The selection and screens used to isolate bacteria are indicated in capitalized and italicized typeface. Relevant genotypes are italicized. *igm* indicates improved growth on minimal medium.

Figure 2 (method chart) is a flow diagram illustrating the method of the present invention of screening a compound for genotoxicity using the instant detector cells and determining the site of action of non-genotoxic compounds by nutritional reversal of genetic titration.

Figure 3 is an illustration of the method for *in vivo* inhibitor identification of specified targets showing the screening of a compound for a known activity.

Figure 4a is a plot of RLU vs. time of strain DPD1715 containing the *ilvB*, *ilvI*, *ilvH*, *ilvG* *relA* and *spoT* mutations and the *recA-LuxCDABE* and being *tolC*⁺, exposed to the DNA damaging agent mitomycin C.

Figure 4b is a plot of RLU vs. time of strain DPD1730 containing the *recA-LuxCDABE* and *tolC*⁺ exposed to the DNA damaging agent mitomycin C.

Figure 5 is a kinetic plot of RLU vs. mitocycin C concentration comparing the sensitivity of tolC- and tolC+ stains to mitomycin C.

Figure 6 is a plot of RLU vs. time for the strain DPD1718 containing the *RecA-LuxCDABE* gene fusion, exposed to varying concentrations of

5 2,4-dichlorophenoxyacetic acid.

Figure 7 is a plot of RLU vs. time for the strain DPD1718 containing the *recA-LuxCDABE* gene fusion, exposed to varying concentrations of sulfometuron methyl.

10 Figures 8(a) and (b) show plots of RLU vs. time for the strain DPD1715 containing the *ilvB*, *ilvI*, *ilvH*, *ilvG* *relA* and *spoT* mutations and the *recA-LuxCDABE* and being tolC+ exposed to varying concentrations of glyphosate.

15 Figure 9 is a plot of RLU vs. time for the strain DPD1730 containing the *recA-LuxCDABE* gene fusion, and the *ilvB* *ilvG* *ilvIH* *relA* *spoT* *tolC+* and *spoT* mutations exposed to varying concentrations of methyl viologen.

Figure 10(a) is a plot of RLU vs. time for the strain DPD1715 containing the *ilvB*, *ilvI*, *ilvH*, *ilvG* *relA* and *spoT* mutations and the *recA-LuxCDABE* and being *tolC+* exposed to varying concentrations of Sulfometuron methyl.

20 Figure 10(b) is a plot of RLU vs. time for the strain DPD1728 containing the *igm* *relA* and *spoT* mutations and the *recA-LuxCDABE* and being *tolC-* exposed to varying concentrations of sulfometuron methyl.

Figure 10(c) is a kinetic plot of bioluminescent measure at 80min vs. concentration of sulfometuron methyl comparing the responses of strains DPD1715 and DPD1728.

25 Figure 11(a) is a plot of RLU vs. time for the strain DPD1718 containing the *recA-LuxCDABE* gene fusion, exposed to varying concentrations of 3-(2-thienyl)-L-alanine in minimal medium.

30 Figure 11(b) is a plot of RLU vs. time for the strain DPD1718 containing the *recA-LuxCDABE* gene fusion, exposed to varying concentrations of 3-(2-thienyl)-L-alanine in Rich LB medium.

Figure 12(a) is a plot of RLU vs. time for the strain DPD1718 containing the *recA-LuxCDABE* gene fusion, exposed to varying concentrations of the oxime carbamate compound OC in minimal medium.

35 Figure 12(b) is a plot of RLU vs. time for the strain DPD1718 containing the *recA-LuxCDABE* gene fusion, exposed to varying concentrations of the oxime carbamate compound OC in Rich LB medium.

Figure 13(a) is a plot of RLU vs. time for the strain DPD1718 containing the *recA-LuxCDABE* gene fusion, exposed to varying concentrations of glyphosate in minimal medium.

Figure 13(b) is a plot of RLU vs. time for the strain DPD1718 containing the *recA-LuxCDABE* gene fusion, exposed to varying concentrations of glyphosate in Rich LB medium.

5 Figure 14 is a plot of RLU vs. time for the strain DPD1718 containing the *recA-LuxCDABE* gene fusion, and the *relA* and $\Delta ilvB$ mutations demonstrating reversal of 3-(2-Thienyl)-L-alanine induced inhibition of bioluminescence by cystein.

Figure 15(a) is a plot of RLU vs. time for strain DPD1718 showing light inhibition by sulfometuron methyl.

10 Figure 15(b) is a plot of RLU vs. time for strain DPD1718 showing restoration of light output after inhibition by sulfometuron methyl as a result of nutritional reversal by the pool comprising the three branched chain amino acids, lysine and histidine.

Figure 16 is a plot of bioluminescent response ratio vs. concentration of thienylalanine contacted with strains transformed with 0245 *ygaH*, *phenA*, and *aroH* genes.

The transformed *E. coli* strains DPD1707, DP1675, and DPD1718 were deposited on 13 February 1997 with the American Type Culture Collection ("ATCC"), international depository located at 12301 Parklawn Drive, Rockville, MD 20852 U.S.A. under the terms of the Budapest Treaty. The strains are designated as ATCC 98328, ATCC 98329, and ATCC 98330 respectively. The designations refer to the accession number of the deposited material.

Applicants have provided a sequence listing in conformity with Rules for the Standard Representation of Nucleotide and Amino Acid Sequences in Patent Applications (Annexes I and II to the Decision of the President of the EPO, published in Supplement No. 2 to OJ EPO, 12/1992) and with 37 C.F.R. 1.821-1.825 and Appendices A and B (Requirements for Application Disclosures Containing Nucleotides and/or Amino Acid Sequences). SEQ ID NO: 1-10 are primer sequences.

30 DETAILED DESCRIPTION OF THE INVENTION

Applicants are the first to recognize that stress promoter-*Lux* gene fusions will be useful in enhancing traditional methods of screening for compound sites of action and the first to develop a detector organism capable of dual functionality in such an assay where the organism is able to denote genotoxic compounds in a first stage and identify site of action in a second assay.

Applicants have developed a method for discovering the site of action of particular xenobiotic compounds by using a bacterial detector cell having a mutation that confers sensitivity to amino acid starvation and containing a gene fusion comprising a genotoxic-sensitive promoter operably linked to a bacterial

bioluminescent reporter gene complex. Typical xenobiotics include chemicals active as herbicides, anticancer agents, antimicrobials and crop protection chemicals. The method has high utility in the agrochemical and pharmaceutical industries for identifying the site of action of compounds and for designing new compounds of similar structure and/or function.

In the method, xenobiotic compounds that are genotoxic interact with the genotoxic-sensitive promoter (driving transcription of the bioluminescent reporter gene complex) and produce an increase in light. Higher concentrations of non-genotoxic xenobiotic compounds may result in the interference of metabolic activity and a decrease in light production.

The present method rapidly determines the site of action of a particular compound via a two part screen involving a uniquely constructed detector cell. The detector cell may possess a *relA* mutation (responsible for diminishing the host cell's response to amino acid starvation) and a *lux* gene fusion comprising a genotoxic-sensitive promoter operably fused to a bacterial *lux* gene complex. In the first stage, detector cells are exposed to specific concentrations of a xenobiotic compound to be screened for and cultures are analyzed for growth inhibition or an increase in bioluminescence. Compounds producing an increase in bioluminescence are discarded as genotoxic. Compounds that result in decreased bioluminescent output relative to the mock-treated control and do not elevate cellular bioluminescence at any tested concentration are subjected to stage two. The second stage uses the detector organism in standard nutritional reversal and genetic titration screens, making use of the bioluminescent gene fusion to identify those nutrients or genes whose supplementation results in prevention of the metabolic interference associated with the xenobiotic compound.

The present method demonstrates for the first time that auxanographic reversal of chemical stressor action can be signaled by restoration of light production of a detector cell strain and that patterns of reversal by defined nutrient pools can define pathways inhibited by the stressor chemical. Advantages of the method include increased numbers of compound screenings per unit of time, increased speed of the biological response, and ease of automation of data collection and processing, while decreasing by a factor of approximately 300 times the amount of compound required for analysis.

The following definitions are used herein and should be referred to for claim interpretation.

The terms "crop protection chemical" and "CPC" refer to compounds having toxic or repellent effect on insects, plant pathogens, or crop-competing plant species known to damage crop plants. CPC's include pesticides (paraquat (methyl viologen), copper sulfate, metidathion), anti-pathogenic compounds such

as fungicides (chlorothalonil, 2-thienylalanine) and profungicides (Oxime Carbamates) or compounds responsible for insect behavior modulation (pheromones, allomones and kairomones), and herbicides referring to compounds having specific or general toxicity to plant species. Typical herbicides include but
 5 are not limited to the class of sulfonylurea herbicides and sulfonanilide herbicides (chlorsulfuron, triasulfuron, metsulfuron-methyl), auxin herbicides (e.g., dicamba, 2-methyl-4-chlorophenoxyacetic acid, picloram, quinclorac, quinmerac), pre-emergence herbicides (metribuzin), and post-emergence herbicides (Clethodim Pendimethalin, oryzalin, dithiopyr, oxadiazon, prodiamine, and
 10 2,4-dichlorophenoxyacetic acid).

“Sulfonylurea herbicides” are defined as N-(heterocyclicaminocarbonyl)-arylsulfonamide-containing herbicidal compounds that inhibit the enzyme acetolactate synthase, such as sulfometuron methyl.

The term “sulfometuron methyl” refers to 2-[[[(4,6-dimethyl-2-pyrimidinyl)amino]carbonyl]amino]sulfonyl]benzoic acid, methyl ester (CAS
 15 registry number 74222-97-2), and is abbreviated as “SM”.

“Acetolactate synthetase” or “ALS” is a key enzyme responsible for branched chain amino acid biosynthesis.

“Glyphosate” will be abbreviated “GP”, has the CAS registry number
 20 1071-83-6 and is a herbicide whose site of action is 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) which catalyzes the conversion of shikimate into anthranilate, a key transformation in plant amino acid synthesis.

The term “thienylalanine-like activity” means any substance, natural or synthetic that has the fungicidal activity of chlorothalonil, 2-thienylalanine.

25 The term “glyphosate-like activity” means any substance, natural or synthetic that acts the interfere with 5-enolpyruvylshikimate-3-phosphate synthase activity.

The term “ALS-inhibiting activity” means” means any substance, natural or synthetic that inhibits the activity of acetolactate synthetase or expression of
 30 the gene encoding acetolactate synthetase.

A “luminescent reporter gene complex” means any reporter gene(s) the products of which result in light production. Examples include but are not limited to the bacterial *lux* genes; the luciferase genes (*luc*), from, for example, the firefly (*Photinus pyralis*) or click beetle (*Pyrophorus plagiophthalmus*); or the gene
 35 encoding the luciferase from the sea pansy (*Renilla reniformis*).

“Site of action” refers to the macromolecular target of a particular stressor or xenobiotic compound. Typical sites of action are specific enzymes in a particular biosynthetic pathway.

The terms "plasmid", "vector", and "cassette" refer to an extra chromosomal element often carrying genes which are not part of the central metabolism of the cell, and are usually in the form of circular double-stranded DNA molecules. Such elements may be autonomously replicating sequences, genome integrating sequences, phage or nucleotide sequences, linear or circular, of a single- or double-stranded DNA or RNA, derived from any source, in which a number of nucleotide sequences have been joined or recombined into a unique construction which is capable of introducing a promoter fragment and DNA sequence for a selected gene product along with appropriate 3' untranslated sequence into a cell.

"Integrand" refers to a bacterial strain into whose chromosome has been inserted a foreign gene fragment.

The term "multiple copies" or "multicopy" as it pertains to the presence of expressible genes in an organisms means a number of copies of the gene that exceeds the normal complement of that gene in the cell.

The terms "transformation" and "transfection" refer to the acquisition of new genes in a cell as a result of the incorporation of nucleic acid. The acquired genes may be integrated into chromosomal DNA or introduced as extrachromosomal replicating sequences. The term "transformant" refers to the product of a transformation.

The terms "promoter" and "promoter region" refer to a sequence of DNA, usually upstream of (5' to) the protein coding sequence of a structural gene, which controls the expression of the coding region by providing the recognition for RNA polymerase and/or other factors required for transcription to start at the correct site. Promoter sequences are necessary but not always sufficient to drive the expression of the gene.

"Genotoxic sensitive promoter" refers to a promoter activated by DNA damage. Examples of these promoters include but are not limited to *recA*, *uvrA*, *lexA*, *umuDC*, *uvrA*, *uvrB*, *uvrC*, *sulA*, *recN*, *uvrD*, *ruv*, *alkA*, *ada*, *dinA*, *dinB*, *dinD*, and *dinF* as well as other promoters which are members of the adaptive response regulon group such as those disclosed by Rupp in *E. coli* and *Salmonella*; Cellular and Molecular Biology [Niedhardt et al., Eds., pp 1190-1220, American Society of Microbiology, Washington, D.C. (1996))] and members of the SOS regulon group disclosed by Walker in *E. coli* and *Salmonella typhimurium*; Cellular and Molecular Biology [Niedhardt et al., Eds., pp 1400-1416, American Society of Microbiology, Washington, D.C. (1996)].

A "fragment" constitutes a fraction of the DNA sequence of the particular region.

“Regulation” and “regulate” refer to the modulation of gene expression controlled by DNA sequence elements located primarily, but not exclusively, upstream of (5' to) the transcription start of a gene. Regulation may result in an “all or none” response to a stimulation, or it may result in variations in the level of gene expression.

The term “operably linked” refers to the fusion of two fragments of DNA in a proper orientation and reading frame to be transcribed into functional RNA.

The term “expression” refers to the transcription and translation to gene product from a gene coding for the sequence of the gene product. In the expression of a gene, a DNA chain coding for the sequence of gene product is first transcribed to a complimentary RNA which is often a messenger RNA and, then, the thus transcribed messenger RNA is translated into the above-mentioned gene product if the gene product is a protein.

“Heightened expression” refers to a gene expression greater than that seen in a mock-treated culture. In the case of *lux* gene fusions, heightened expression is indicated by an increase in bioluminescence above background levels that is characterized by a temporal delay that allows for increased transcription and subsequent translation.

The terms “stressor” or “insult” refer to a chemical agent or physical treatment that results in suboptimal growth of an organism. Stressors may include, but are not limited to, chemicals (such as herbicides, crop protection chemicals, environmental pollutants, heavy metals), physical treatments such as changes in temperature, changes in pH, agents producing oxidative damage or DNA damage (such as from UV exposure), anaerobiosis, biological insults such as the introduction of other life forms (viruses, bacteria, etc.) into the bacterial culture, or changes in nutrient availability. Additionally, stressors may include naturally-occurring compounds such as L-valine, galactose-phosphate, 2-ketobutyrate. A “stressor target” means a specific macromolecular target inhibited by a specific stressor.

The terms “xenobiotic compound” and “xenobiotic chemical” refer to any stressor chemical which does not typically occur in nature. Typical xenobiotics of interest in the present invention include those useful as herbicides, pesticides, fungicides or any other xenobiotic capable of interfering with a specific metabolic site of action.

The term “bioluminescence” refers to the phenomenon of light emission from a living organism.

“Bioluminescent positive phenotype” refers to a phenotype displaying an increase in light production by a detector cell containing a *lux* gene fusion.

The term "baseline luminescence" means the amount of light produced by a cell having a bioluminescent positive phenotype in an unstressed metabolic state.

The term "*lux* gene complex" refers to the *lux* structural genes which include *luxA*, *luxB*, *luxC*, *luxD* and *luxE* and which are responsible for the phenomenon of bacterial bioluminescence. A *lux* gene complex might include all of the independent *lux* genes, acting in concert, or any subset of the *lux* structural genes so long as *luxA* and *luxB* are part of the complex.

"Gene fusion" is a hybrid DNA fragment comprising a regulatory signal essential for transcription (referred to as a promoter) fused to at least one structural gene sequence coding for a specific polypeptide.

The term "*lux* gene fusion" means the fusion of the *lux* gene complex with a suitable stressor-sensitive promoter. "*recA-LuxCDABE*" refers to the specific fusion of the genotoxic sensitive promoter *recA* fused to the bacterial *Lux* gene complex.

The term "*ilvBN*" refers to the structural genes encoding, respectively, the large and small subunits of the heterotetrameric ALS I-EC 4.1.3.18.

The term "*ilvGM*" refers to the structural genes encoding, respectively, the large and small subunits of the heterotetrameric ALS II-EC 4.1.3.18. *ilvGM* is cryptic in *E. coli* K-12 Δ *ilvB*-a mutation that deletes part of the *ilvB* gene.

The term "*ilvIH*" refers to the structural genes encoding, respectively, the large and small subunits of the heterotetrameric ALS III-EC 4.1.3.18. *ilvIH* is cryptic in laboratory strains of *Salmonella typhimurium*.

The term "*pheA*" refers to the structural gene encoding the bifunctional polypeptide that displays chorismate mutase (EC 5.4.99.5) and prephenate dehydratase (EC 4.2.1.51) activities.

The term "*relA*" refers to the structural gene encoding the ATP:GTP 3'-pyrophosphotransferase I-EC 2.7.6.5.

The term "*spoT*" refers to the structural gene encoding the ATP:GTP 3'-pyrophosphotransferase II-EC 2.7.6.5.

The term "*aroA*-" refers to the structural gene encoding enolpyruvylshikimate phosphate synthase-EC 2.5.1.19.

The term "*aroH*-" refers to the structural gene encoding DHAP(tryptophan repressible and feed back inhibitable) synthase-EC4.1.2.15.

The term "*tolC*+" refers to the structural gene encoding an outer membrane porin needed for the efflux of many xenobiotics pumped out by a variety of membrane translocases.

The term "*glk*-" refers to the structural gene encoding glucokinase-EC2.7.1.2.

The terms "detector organism", "detector bacteria", and "detector cell" refer to an organism which contains a gene fusion consisting of a genotoxic-sensitive promoter fused to a luminescent reporter gene or gene complex.

5 The term "non-bioluminescent parent" of the detector cell is a bacterial strain into which a light producing genetic cassette has not been introduced.

"Genetic titration" refers to an alteration of the genetic makeup of a microbe such that the levels of a macromolecular target are elevated to a point whereby they overcome the action of a stressor. Within the context of the present invention genetic titration will involve a process of screening for biochemical
10 targets of compounds

where a host organism is transfected with a suitable genomic library, transformants are screened for growth in the presence of the compound and portions of the library conveying resistance to the compound are isolated and identified.

15 "Nutritional reversal" refers to the addition of a nutrient to a culture contacted with a stressor such that the biological output of the culture is restored to the unchallenged level.

"Nutrient" refers to an end product of a biochemical pathway or a compound readily converted to a pathway end product. Typical nutrients are
20 amino acids, vitamins, bases or sugars. Vitamins are readily converted to cofactors which are pathway end products; similarly bases are readily transformed *in vivo* into nucleotide triphosphates.

The term "auxanography" means the diagnostic and systematic administration of nutrient pools to determine the pathway blocked in a
25 microorganism as described in Davis, R. W., D. Botstein And J. R. Roth. A Manual For Genetic Engineering: Advanced Bacterial Genetics. X+254p. Cold Spring Harbor Laboratory: Cold Spring Harbor, N.Y., U.S.A., 1980.

The term "Relative Light Unit" is abbreviated "RLU" and refers to a measure of light emission as measured by a luminometer, calibrated against an
30 internal standard unique to the luminometer being used.

DETECTOR CELLS AND NONBIOLUMINESCENT STRAINS

Host cells suitable in the present invention include any cell capable of expression of the *lux* gene fusion where prokaryotic cells are preferred and where members of the enteric class of bacteria are most preferred.

35 Enteric bacteria are members of the family *Enterobacteriaceae* and include such members as *Escherichia*, *Salmonella*, and *Shigella*. They are gram-negative straight rods, 0.3-1.0 X 1.0-6.0 mm, motile by peritrichous flagella (except for *Tatumella*) or nonmotile. They grow in the presence and absence of oxygen and grow well on peptone, meat extract, and (usually) MacConkey's media. Some

- grow on D-glucose as the sole source of carbon, whereas others require vitamins and/or mineral(s). They are chemoorganotrophic with respiratory and fermentative metabolism but are not halophilic. Acid and often visible gas is produced during fermentation of D-glucose, other carbohydrates, and polyhydroxyl alcohols. They are oxidase negative and, with the exception of *Shigella dysenteriae* O group 1 and *Xenorhabdus nematophilus*, catalase positive. Nitrate is reduced to nitrite (except by some strains of *Erwinia* and *Yersina*). The G + C content of DNA is 38-60 mol% (T_m , Bd). DNAs from species within most genera are at least 20% related to one another and to *Escherichia coli*, the type species of the family. Notable exceptions are species of *Yersina*, *Proteus*, *Providencia*, *Hafnia* and *Edwardsiella*, whose DNAs are 10-20% related to those of species from other genera. Except for *Erwinia chrysanthemi*, all species tested contain the enterobacterial common antigen (Bergey's Manual of Systematic Bacteriology, D. H. Bergey et al., Baltimore: Williams and Wilkins, 1984).
- Particularly useful in the present invention are *E. coli* and members of the genus *Salmonella* and those enterics for which the *recA* gene and its upstream promoter region has been identified.

Membrane Mutations

- Host cells of the present invention optionally may contain mutations that will facilitate the screening process. The appropriate bacterial strain with which to test the effects of a chemical is one whose growth is affected by that chemical. Hence, the chemical of interest must be able to enter the cell, be retained in the cell, and interact with target molecules of the cellular machinery. Various mutations of *E. coli* are known to affect permeation into and accumulation within the cell. Strains carrying mutant alleles of *rfa* (Ames, B. N., F.D. Lee, and W. E. Durston, *Proc. Nat. Acad. Sci. USA*, 70(3): p. 782-786, (1973)), *envA* (Young, K. and L. L. Silver, *J. Bacteriol.*, 173: p. 3609-3614, (1991)), *imp* (Sampson, B. A., R. Misra, and S. Benson, *Genetics*, 122: p. 491-501, (1989)), *lpp*, (Giam, C. Z., S. Hayashi, and H. C. Wu, *J. Biol. Chem.*, (259): p. 5601-5605, (1984)) or *surA* (Tormo, A., M. Almiron, and R. Kolter, *J. Bacteriol.*, (172): p. 4339-4347, (1990)) have increased sensitivity to a variety of chemicals. Destruction of efflux pumps, with mutations such as *emr* (Ma, D., et al., *Mol. Microbiol.*, 16: p. 45-55, (1995)), or *acrAB* (Paulsen, I.T., et al., *Mol. Micro.*, 19: p. 1167-1175, (1996)), or the channels they use, with mutations such as *tolC* (Schnaitman et al., *J. Bacteriol.*, 172 (9), pp 5511-5513, (1990)), also result in increased chemical sensitivity. Moreover, it is contemplated that genetic titration's in conjunction with genomics will permit the development of method for the identification of such sensitizing mutations which can then be integrated in to an appropriate detector cell background. In some instances, the target macromolecule of a chemical may be

intrinsically resistant to the action of that chemical. For example, *E. coli* has two isozymes of the enzyme acetolactate synthase, one of which has a poor binding affinity for the sulfonylureas herbicides. Mutations which destroy the function of *ilvBN*, encoding the resistant isozyme, result in a strain with greatly increased susceptibility to growth inhibition by sulfonylureas that target acetolactate synthase (LaRossa, R. A. and D. R. Smulski, *J. Bacteriol.*, 160: p. 391-394, (1984)). An appropriate host strain of *E. coli* or other bacteria may be constructed to carry a known mutation or combinations of mutations. Furthermore, an appropriately sensitive strain may also be found by screening for growth inhibition following mutagenesis by transposon insertion or chemical or physical treatments.

Mutations Conferring Stress Sensitivity

Detector cells of the present invention optionally may contain mutations that will convey sensitivity to a particular stress to be screened for. The *relA* mutation, for example, prevents the cell from responding to amino acid starvation. Other mutations that will be useful in stress sensitivity include, for example, mutations that result in sensitivity to various anticancer drugs and compounds that cause oxidative stress. It is known that many anticancer drugs interfere with DNA replication while compounds that cause oxidative stress may be useful in controlling fungal pathogens of crops and bacterial pathogens of humans or animals. Mutations in genes conveying sensitivity to stresses are preferred, including but not limited to, mutant genes selected from the group consisting of *cya*, *crp*, *spoT*, *arcAB*, *envZ*, *ompR*, *marR*, *earAB*, *fur*, *oxrG*, *fruR*, *rpoS*, *rpoE*, *creB*, *creC*, *glnG*, *glnL*, *glnB*, *glnD*, *glnF*, *phoB*, *phoP*, *phoQ*, *phoR*, *phoU*, *rpoH*, *lexA*, *recA*, *lrp*, *soxRS*, *oxyR*, *fnr*, *atbR*, *ada*, and *relA* where the *relA* mutation is most preferred. Moreover, genetic titration, may indicate unexpected roles for regulatory genes. Such discovery, coupled with inactivation of the regulatory locus, may optimize the sensor strains.

Within the context of the present invention strains generally contained mutations such that only a single ALS (I or III) sensitive to sulfonylurea herbicides was expressed. Such a sensitive host may either be screened from wild type after standard transposon, chemical (e.g., HNO_2 and NH_2OH), UV, intercalating dye (e.g., acridine dyes) or other mutagenesis protocols have generated the appropriate hypersensitive mutations or can be constructed by combining mutations that together yield the desired sensitivity. For a review of the methods of mutagenesis, see, for example, Thomas D. Brock in Biotechnology: A Textbook of Industrial Microbiology, Second Edition (1989) Sinauer Associates, Inc., Sunderland, MA.

Stress Sensitive Reporter; *Escherichia coli* and *Salmonella*; Cellular and Molecular Biology (Niedhardt et al. Eds., American Society of Microbiology, Washington, D.C. (1996))].

The detector cell of the present invention optionally may also contain a stress-sensitive reporter for the detection of particularly desirable or undesirable characteristics of the stressor compound to be screened. Typically, stress-sensitive reporters are comprised of a stress-sensitive promoter operably linked to a suitable reporter element. The promoter must be chosen so as to be expressible within the specific detector cell desired. Typically in bacterial detector cells, the promoters will be chosen from stress-inducible bacterial promoters. Examples of stress-inducible promoters suitable in the present invention are those responsive to chemicals, environmental pollutants, heavy metals, xenobiotics, changes in temperature, changes in pH as well as agents producing oxidative damage, DNA damage, anaerobiosis, changes in nitrate availability or pathogenesis. Specific examples of suitable bacterial stress-promoters include, but are not limited to, those sensitive to protein damage such as the heat shock genes (*grpE*, *dnaK*, *lon*, *rpoD*, *groESL*, *lysU*, *htpE*, *htpG*, *htpI*, *htpK*, *clpP*, *clpB*, *htpN*, *htpO*, and *htpX*), those sensitive to DNA damage such as those controlled by the SOS regulatory circuit (*recA*, *uvrA*, *lexA*, *umuDC*, *uvrA*, *uvrB*, *uvrC*, *sulA*, *recN*, *uvrD*, *ruv*, *dinA*, *dinB*, *dinD*, and *dinF*), those sensitive to oxidative damage (*katG*, *ahp*, *micF*, *sodA*, *nfo*, *zwf*, and *soi*), those sensitive to membrane damage (*fabA*), those sensitive to amino acid starvation and under the control of the *relA*, or *spoT* regulatory genes (*his*, *ilvBN*, *ilvGMED*, and *thrABC*), those sensitive to carbon starvation and under the control of the *cya*, and *crp* regulatory genes (*lac*, *mal*, and *gal*), those sensitive to phosphate starvation under the control of the *phoB*, *phoM*, *phoR*, and *phoU* regulatory genes (*phoA*, *phoBR*, *phoE*, *phoS*, *aphA*, *himA*, *pepN*, *ugpAB*, *psiD*, *psiE*, *psiF*, *psiK*, *psiG*, *psiL*, *psiJ*, *psiN*, *psiR*, *psiH*, *phiL*, *phiO* *ara*, *tna*, *dsd*, and *hut*), and those sensitive to nitrogen starvation under the control of the *glnB*, *glnD*, *glnG*, and *glnL* regulatory genes (*glnA*, and *hut*). Preferred within the context of the present invention are those stress-inducible promoters sensitive to genotoxicity or DNA damage under the control of the SOS regulatory circuit where the *recA* gene is most preferred.

Reporter genes suitable for fusion to the stress inducible promoter are structural genes under the control of such a promoter and able to report a detectable signal. Many bacterial reporters such as *lacZ*, *galK*, *xylE*, *luc*, *luxAB*, *luxCDABE*, *phoA*, *uidA* (GUS), *cat*, *npt-II*, *SUC2* and ubiquitin are known in the art (Miller, J. H., A Short Course in Bacterial Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, pp. 63-67). Of particular use in the present invention are bioluminescent reporter genes including but not limited

to the bacterial *lux* genes; lucifersae genes (*luc*) from, for example, the firefly, *Photinus pyralis*, or click beetle, *Pyrophorus plagiophthalmus*; or the gene encoding the luciferase from the sea pansy, *Renilla reniformis*. Because its gene products function well in *E. coli* under a wide range of temperatures, most preferred is the promoterless *Photorhabdus luminescens luxCDABE* gene complex obtained from the pCGLS1 plasmid containing the *lux* gene complex. This complex is fully described by Rosson, R. A., in PCT International Application WO 93/03179 (1993).

Bacterial bioluminescence as produced by the *luxCDABE* gene complex is a phenomenon in which the products of 5 structural genes (*luxA*, *luxB*, *luxC*, *luxD* and *luxE*) work in concert to produce light. The *luxD* product generates a 14 carbon fatty acid from a precursor. The 14 carbon fatty acid is activated in an ATP dependent reaction to an acyl-enzyme conjugate through the action of the *luxE* product which couples bacterial bioluminescence to the cellular energetic state. The acyl-enzyme (*luxE* product) serves as a transfer agent, donating the acyl group to the *luxC* product. The acyl-*LuxC* binary complex is then reduced in a reaction in which NADPH serves as an electron pair and proton donor reducing the acyl conjugate to the C¹⁴ aldehyde. This reaction couples the reducing power of the cell to bacterial light emission. The light production reaction, catalyzed by luciferase (the product of *luxA* and *luxB*), generates light. The energy for light emission is provided by the conversion of aldehyde to fatty acid and FMNH₂ oxidation, providing another link between light production and the cellular energy state.

The usefulness of *luxCDABE* are limited by the inherent thermolability of the protein products of these genes. The temperature requirement of this reporter system has limited overlap with the need to grow bacteria rapidly in defined media. Applicants have solved this problem by using *luxCDABE* that encode protein products capable of functioning in the desired temperature range (28-42°C).

A multiplicity of strains were engineered, each having a specific genotype useful for the specific site of action screen desired. Construction of the strains is reviewed in Figure 1 and the genotypes are summarized in Table 1 in the GENERAL METHODS. All strains engineered to contain the *recA-Lux-CDABE* gene fusion functioned as detector cells. Detector cells may contain only the gene fusion, or optionally may possess other mutations affecting membrane permeability or stressor sensitivity.

Useful strains possess a variety of genotypes including where the expression or suppression of the *ilvB*, *relA*, *tolC*, *igm*, *spoT* and *ilvG* genes were used to give useful detector cells. Methods of strain construction are well known

in the art and use the basic elements of molecular biology and microbiology fully discussed in Sambrook, J. et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press (1989); *Escherichia coli* and *Salmonella*; Cellular and Molecular Biology (Niedhardt et al. Eds., American Society of Microbiology, Washington, D.C. (1996))].

Culture Conditions:

Typically, cells are grown at 37 °C in an appropriate media. Preferred growth medium in the present invention are common defined media such as Vogel-Bonner medium (Davis et al., *Advanced Bacterial Genetics*, (1980), Cold Spring Harbor, NY: Cold Spring Harbor Laboratory). Other defined or synthetic growth media may also be used and the appropriate medium for growth of the particular microorganism will be known by one skilled in the art of microbiology or fermentation science. In some instances rich or complete media such as NB (Nutrient broth) are used.

Suitable pH ranges for bacterial growth are between pH 5.0 to pH 9.0, where pH 6.0 to pH 8.0 is preferred as the initial condition.

Growth of the bacterial cells in liquid medium allows a uniform population of cells to be stressed at various growth phases such as early log phase, mid log phase, late log arithmetic phase, or stationary phase. Stress is the condition produced in a cell as the result of exposure to a cellular insult or stressor. This cellular stress may be caused by any substance or change in the cellular environment that results in an alteration of normal cellular metabolism in a bacterial cell or population of cells. The addition of chemicals such as herbicides, crop protection chemicals, environmental pollutants, or heavy metals to the growth media can cause such a stress. Additionally, changes in temperature, changes in pH, agents producing oxidative damage or DNA damage (such as from UV exposure), anaerobiosis, or changes in nitrate availability are insults that may cause stress as well.

STRESSOR XENOBIOTICS AND CROP PROTECTION CHEMICALS

The present method is useful for identifying the site of action for a variety of xenobiotic chemicals and particularly those compounds useful as crop protection chemicals. The instant method is useful for determining the site of action of compounds selected from but not limited to sulfonylurea herbicides such as sulfometuron methyl, glyphosate, the profungicides such as the oxime carbamates, oxidants such as methyl viologen (paraquat), hormonal herbicides such as 2,4-dichlorophenoxyacetic acid, and fungicides such as 2-thienylalanine. Any chemical that negatively impacts bacterial metabolism can be analyzed in this manner. Thus, not only antibacterials, but also antivirals, anticancer agents, anticorrosives, disinfectants and crop protection chemicals may be analyzed with

this invention since the site of action of many such compounds are often enzymes or cofactors shared by bacteria and target organisms. Moreover, products of normal metabolism whose accumulation is detrimental can be analyzed with these methods.

5 SITE OF ACTION SCREENS

Determination of the site of action of various stressors and xenobiotic compounds by the present method proceeded using the complementary screens of nutritional reversal and genetic titration.

Nutritional Reversal

10 The above defined media provides those nutrients (inorganic compounds and carbons sources) necessary and sufficient for the growth of wild type *E. coli*. *E. coli* detector cells (containing the *recA-Lux* gene fusion) grow in these media converting the nutrients into organic chemicals required for cell growth and metabolism. If a stressor chemical interferes with one of the biosynthetic
15 processes, the cellular metabolism and the bioluminescence of a culture will be reduced or eliminated.

The present method of nutritional reversal involves first screening compounds for the ability to depress bioluminescence of the detector organism containing the *recA-LUX* gene fusion. Next, panels of cultures are initiated to test
20 which nutrient will reverse the growth inhibition as indicated by a reversal of bioluminescent depression. Stressor chemicals meeting this criteria are defined as nutritionally reversed. The specific pathway affected by a stressor chemical is then determined by auxanographic analysis. Supplementation with biosynthetic intermediates from the identified pathway to determine which intermediates
25 obviate the depression of bioluminescence may be used to define the target within the pathway. Modulation by non-pathway nutrients (Van Dyk and LaRossa, pp. 123-130 and LaRossa et al, pp 109-121 both in Biosynthesis of Branched Chain Amino Acids, Barak et al., eds. Balaban Publishers, Weinheim, FRG, 1990) can also help to define the macromolecular target of the stressor chemical.

30 Genetic Titration

The genetic titration protocol is predicated upon the fact that chemical stressor action can be overcome by increasing the intracellular concentration of the macromolecular target above that normally found. In this manner the inhibitor action is titrated out and cells grow under conditions that normally inhibit the
35 growth of the wild type. The means of increasing the macromolecular target could be through the selection of genetic duplications (e.g., Anderson and Roth, *Ann Rev Microbiol.*, 31:473-505 (1977); Wahl et al., *J Biol Chem*, 254: 8679-89 (1979), Alt et al., *J Biol Chem*, 253: 1357-70 or through the selection of high level constitutive regulatory mutants (Roth et al., *J. Mol. Biol.* 22:305-323 (1966);

Hartman, Ames, Anton Yanofsky Calvo et al., *J. Bacteriol.* 97:1272-1282 (1969), Calvo et al., *Genetics*, 61:777-787 (1969)). These methods, however, lead to rather laborious analyses to define the overexpressed gene product. A preferred embodiment is that pioneered by Falco [Falco and Dumas, *Genetics* 109:21-35 (1985)] and Rine [Rine et al., *PNAS USA* 80:6750-6754 (1983)] in yeast in which gene amplification is created by (a) construction of genomic libraries in high copy number autonomously replicating plasmids and (b) the introduction of such plasmid libraries into a suitable host strain creating a catalog of mero-multiploids [LaRossa, (1996), in *Escherichia coli* and *Salmonella*; Cellular and Molecular Biology (Niedhardt et al. Eds., pp 1400-1416, American Society of Microbiology, Washington, D.C. (1996))]. In the next stage, (c), the meromultiploids conferring resistance to the chemical stressor are identified by selection for colonies that grow above the compound's MIC (minimal inhibitory concentration). Such methodology has been recently extended to *E. coli* (Chatterjee and Sternberg, *PNAS USA* 92:8950-8954 (1995)).

Prevention Of Inhibitor Action By Partial Genome Amplification.

In addition to the techniques of nutritional reversal and genetic titration it is contemplated that present detector cells may be employed to identify compounds on the basis of the prevention of inhibitor action. For example, the present application exemplifies the incorporation of several genomic fragments suspected of or known to confer growth resistance to thienylalanine into a bioluminescent or other tester strains by transformation selecting for ampicillin resistance. The resultant bioluminescent strains, along with negative controls in which the multicopy plasmid vectors lacking an insert were used for transformation, were exposed to various concentrations of thienylalanine and kinetic plots of bioluminescence versus time were obtained (Example 9). As demonstrated in Example 9, clone *o245 ygaH* conferred the greatest degree of protection from thienylalanine while other clones confer intermediate levels of resistance. It is evident from this Example that (a) cross resistance can be discovered by simply replacing thienylalanine with other compounds in the test (indeed disk diffusion assays have revealed cross resistance conferred by *o245, ygaH*, to several other amino acid analogs including those identified in table 12) and; (b) other chemicals hitting a specific target can be identified by asking if particular target specifying gene in multiple copies confers inhibitor resistance. In this manner new inhibitors of acetolactate synthase have been revealed).

Membrane Alterations

In another embodiment it is contemplated that the present detector cells may be modified to incorporate disrupted membrane proteins which in turn may be exploited to identify compounds having a specific biological activity. For

example, genetic titration with SM identified *tolC* as a resistance determinant. Similarly titration with GP recognized *yhhTS*, thienylalanine recognized *o245 ygaH*, acivicin recognized *yedA* and mitomycin C recognized *mdfA* as resistance determinants (Examples 7-9). Each of these genes encodes a predicted membrane protein. It is axiomatic that disruption of these cloned genes and incorporation of the disruptant into the *E. coli* chromosome can be achieved by standard techniques without undo experimentation. The utility of such disruptants can be readily assessed by bioluminescent assays that determine the doses that reduce light output by a factor of 2. These disrupted genes will be useful for a plethora of sensitive bioassays.

Discussion of the Preferred Embodiments

A preferred embodiment of the present method is illustrated in Figure 2. A detector organism (1) having a *recA-luxCDABE* fusion and a *relA* mutation (inhibiting the cell's response to amino acid starvation) is exposed to a battery of compounds (stressors) (2) to be screened for crop protection activity. Compounds producing an increase in light from the detector cell are discarded as genotoxic. Compounds that slow cell growth but do not produce an increase in light are subjected to nutritional reversal screens or genetic titration screens to determine the site of action of the compound. Where nutritional reversal is used, detector cells are grown in a minimal medium supplemented with a multiplicity of different nutrient pools, each pool composed of a different mixture of nutrients (3). The pool supplying the amino acid necessary to reverse metabolic inhibition is detected by the recovery of light production by the bioluminescent gene fusion (4).

The skilled person will appreciate that advances in genomics make a number of related embodiments possible. For example, into the detector organism described in the preceding paragraph an ordered set of overlapping, high copy number plasmids is placed such that each contain different segments of the *E. coli* chromosome. This set of resulting strains may be used in a genetic titration screen of a chemical stressor to identify those chromosomal regions that upon amplification restore bioluminescent output to uninhibited levels. Thus, genetic titration may be performed in alternative selection and screening modes.

In another embodiment, detector cells containing the *recA-LUX* gene fusion were constructed by transformation of suitable hosts with the chimera according to standard methods (Sambrook *supra*). The transformants possessed a variety of mutations including *ilvB*, *relA* and *tolC*. Detector cells were constructed so as to contain one or more of these mutations. These detector cells emitted a baseline luminescence that was altered by the exposure to various xenobiotics.

The sensitivity of the detector cell to genotoxic agents was examined by exposing the cell to mitomycin C. Moderate levels (0.3-20 $\mu\text{g/mL}$ for a *tolC*⁺ strain, 0.3-1.25 $\mu\text{g/mL}$ for a *tolC* derivative) of mitomycin C resulted in an increase in baseline luminescence. High levels (>2.5 $\mu\text{g/mL}$ in a *tolC* strain) of mitomycin C resulted in a decrease in luminescence (Figures 4 and 5).

Detector cells containing a variety of mutations were treated with 4 different herbicides (SM, MV, GP and 2,4-D) to determine the effect of the compounds on bioluminescent output of the detector cell. In each case kinetic plots indicate a dose-dependent decrease in light emission in response to the herbicides. Data illustrating the effect is seen in Figures 6-9).

Detector cells containing the *tolC* mutation were exposed to varying concentrations of SM and mitomycin C to determine the effect of the mutation on the sensitivity of the assay. As is noted in Tables 2 and 3 and Figures 4 and 10 and 4 the *tolC* mutation not only enhanced the responsiveness of the detector cell to the lipophilic SM but also to mitomycin C. These tests demonstrated the utility of the *tolC* mutation as a component of the detector cell.

The site of action of two CPC's were analyzed using a detector cell comprising the *recA-LuxCDABE* fusion. Using the bioluminescent detector cells, nutritional reversal was applied to each of the compounds to determine which nutrient would reverse the growth inhibiting effects of the compound. As illustrated in Tables 2 and 3, cysteine metabolism was identified as the potential affected site for cells treated with the oxime carbamate compound while phenylalanine metabolism was the identified site of action for the cells treated with thienylalanine.

Finally, detector cells harboring *ilvB*, *relA* and *tolC* alleles were used in genetic titration assays to determine the site of action of the SM, 2-thienylalanine and GP. The detector cells were transformed with *E. coli* cDNA libraries and transformants were screened for resistance to SM as indicated by changes in bioluminescence of the transformants. Resistant colonies were picked and the plasmids isolated, sequenced and analyzed for genes encoding for possible targets. In this fashion the *ilvBN* and *ilvIH* genes were identified and confirmed as the genes encoding ALS, the target for SM. In similar fashion the *aroA*, the known target for GP encoding EPSPS, was selected. The *aroH* gene was obtained in a selection for 2-thienylalanine resistant clones.

In another alternate embodiment describing *in vivo* inhibitor identification of specified targets, the present detector cells may also be utilized in a method to screen for compounds where the site of action is known. Such an embodiment is illustrated in Figure 3. It is contemplated that a detector organism may be constructed to include not only the stress promoter-*luxCDABE* gene fusion, but

also a plasmid expressing the gene target of the compound to be screened. For example, it is known that the gene target for GP is 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) encoded by the *aroA* gene, and plasmids have been constructed so as to express the EPSPS gene product. Compounds to be screened would be exposed to a standard detector cell (1) and the modified detector cell (2) containing the expressible gene target (3) at suitable concentrations. No addition of compounds (4) will give a baseline luminescence in both detector cells. Addition of compounds that inhibit growth (5) will produce a decrease in luminescence in both detector cells. However, where exposure of the standard detector cell (1) to a glyphosate-like molecule (6) will produce a decrease in light production, exposure of the same compound to the modified detector cell (2) will give a smaller or no decrease in light production. The compound was genetically titrated by the expression of the gene target in the modified detector cell(2).

It will be appreciated by one skilled in the art that the *E. coli aroA* gene may be substituted with plant or other bacterial *aroA* homologues. Moreover, it is contemplated that chemistry for any target of interest can be identified in this manner. The method of *in vivo* inhibitor identification of specified targets here exemplified could be used to identify many useful compounds that include, but are not limited to, new crop protection chemistries, antibacterial chemistries, antifungal chemistries, anticancer chemistries, anti-viral chemistries, chemistries preventing biofilm formation and anticorrosives.

The method of *in vivo* inhibitor identification of specified targets may also be used to detect inhibitors of ALS III, for example. DPD1718 could be transformed with pBR322 selecting for ampicillin or tetracycline resistance yielding strain pBR322/DPD1718. Similarly DPD1718 may be transformed with an *ilvIH* containing plasmid selecting for ampicillin resistance yielding strain pIlvIH/DPD1718 having an ALS III phenotype. These two transformed strains may then be used to screen for specific inhibitors of ALS III since the titer of ALS III will be much greater in strain pIlvIH/DPD1718 than in pBR322/DPD1718.

Expected results follow:

Chemical	Relative MLC*	
	pIlvIH/DPD1718	pBR322/DPD1718
Non-ALS toxicant	1	=1
ALS III inhibitor	>1	=1

*Minimal Lumino - Inhibitory Concentration

It is contemplated then that one can score other growth related phenotypes or growth itself to identify novel compounds interfering with EPSP, ALS or other desired cellular functions.

5 The above embodiment is more fully exemplified in Examples 7-9 which demonstrate how the introduction of genes conveying resistance to Mitomycin C, Acivicin and Thienylalanine into a *recA-LUX* detector cell may be used to screen for compounds having to Mitomycin C, Acivicin and Thienylalanine activity respectively.

10 In another embodiment the *recA-LUX* detector cell is used as a means for screening compounds for mutagenicity. The efficacy of the present SOS regulated bioluminescent gene fusions is seen in the comparison of fusion containing detector cells as indicators of compound mutagenicity as compared with the standardized Ames test (Example 10). Compounds chosen at random which tested positive in the standard Ames test were confirmed as mutagenic by giving
15 a 'lights-on' response in bioluminescent detector cells.

The present invention is further defined in the following Examples. It should be understood that these Examples, while indicating preferred embodiments of the invention, are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the
20 essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions.

EXAMPLES

GENERAL METHODS

25 Procedures for phosphorylations, ligations and transformations are well known in the art. Techniques suitable for use in the following examples may be found in Sambrook, J. et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press (1989).

30 Materials and methods suitable for the maintenance and growth of bacterial cultures are well known in the art. Techniques suitable for use in the following examples may be found in Manual of Methods for General Bacteriology (Phillipp Gerhardt, R. G. E. Murray, Ralph N. Costilow, Eugene W. Nester, Willis A. Wood, Noel R. Krieg and G. Briggs Phillips, eds), American Society for Microbiology, Washington, DC. (1994) or Thomas D. Brock in Biotechnology: A
35 Textbook of Industrial Microbiology, Second Edition (1989) Sinauer Associates, Inc., Sunderland, MA. Standard bacterial genetic protocols can be found in Miller, J. H., Experiments in Molecular Genetics, Cold Spring Harbor Laboratory (1972); Miller, J. H., (1992) A Short Course in Bacterial Genetics, Cold Spring Harbor Laboratory Press; Silhavey, T. J., Berman, M. L., and Enquist, L. W.,

Experiments with Gen Fusions (1984), Cold Spring Harbor Laboratory, and Davis, R. W., Botstein, D. and Roth, J. R. (1980), A Manual for Genetic Engineering-Advanced Bacterial Genetics, Cold Spring Harbor Laboratory. All reagents and materials used for the growth and maintenance of bacterial cells were
 5 obtained from Aldrich Chemicals (Milwaukee, WI), DIFCO Laboratories (Detroit, MI), GIBCO/BRL (Gaithersburg, MD), or Sigma Chemical Company (St. Louis, MO) unless otherwise specified.

The meaning of abbreviations is as follows: "h" means hour(s), "min" means minute(s), "sec" means second(s), "d" means day(s), "mL" means
 10 milliliters, "L" means liters.

Bioluminescence was measured in a luminometer, model ML3000 (Dynatech Laboratories, Chantilly, VA) according to the manufacturer's instructions.

Crop protection chemicals used in the following examples were
 15 sulfometuron methyl [obtained from DuPont Agricultural Products, Wilmington, DE], glyphosate [obtained from Sigma], methyl viologen [obtained from Sigma] 2-Thienylalanine [obtained from Aldrich], compound OC, member of the class of oxime carbamates having profungicide activity was prepared by DuPont Agricultural Products. Mitomycin C and acivicin was obtained from Sigma.

20 Stock solutions were prepared as follows:

2-thienylalanine - (10 mg/mL) in water
 sulfometuron methyl - (3.6 mg/mL) in 0.01 N NaOH
 glyphosate - (5 mg/mL) in water
 compound OC - (10 mg/mL) in DMSO (dimethylsulfoxide)
 25 methyl viologen - (2 mg/mL) in water
 mitomycin C - (1 mg/mL) in water
 acivicin 10 mg/ml in water.

Strains

The transformed *E. coli* strains DPD1707, DP1675, and DPD1718 were
 30 deposited on 13 February 1997 with the American Type Culture Collection ("ATCC"), international depository. The strains are designated as ATCC 98328, ATCC 98329, and ATCC 98330 respectively.

Additional strains of detector cells useful in the determination of CPC sites of action are given in Table 1, below.

35

TABLE 1

Strain	Genotype
CU847	<i>ilvB2101 ara thi</i> Δ (<i>pro-lac</i>), [from E. Umbarger, Purdue University]
DPD1678	as CU847 but Δ <i>relA251::Kan</i>
DPD1675	as CU847 but <i>tolC::miniTn10</i>
DPD1690	as DPD1675 but Δ <i>relA251::Kan</i>
DPD1012	as DPD1690 but <i>igm</i> (improved growth on minimal medium, an unmapped mutation)
DPD1013	as DPD1690 but <i>igm</i> (improved growth on minimal medium, an unmapped mutation)
RK4988	<i>F' araD139</i> Δ (<i>lacIPOZYA</i>) <i>U169 strA thi? non gyrA</i> Δ (<i>ilvB-uhpA</i>)2089 [From R. Kadner via Ying-Ying Chang, University of Illinois]
DPD1692	as <i>Kan</i> resistant, lactose non-utilizing
DPD1680	as RK4988 but <i>tolC::miniTn10</i>
DPD1693	as DPD1680 but Δ <i>relA251::Kan</i>
FD1062	Hfr <i>ara-14 ilvI614 ilvH612</i> λ - <i>glyA18 relA1 spoT1 ilvB619 bglR20 rbs-5::Tn5 ilvG468</i> (<i>IlvG+</i>) <i>thi-1</i> , [from Bob Lawther, University of South Carolina, reference: Babczinski, P. and T. Zelinski, (1991) <i>Pestic. Sci.</i> 31: 305-323]
DPD1682	as FD1062 but <i>tolC::miniTn10</i>
DPD1010	as DPD1682 but <i>igm</i> (improved growth on minimal medium, an unmapped mutation)
DPD1011	as DPD1682 but <i>igm</i> (improved growth on minimal medium, an unmapped mutation)
DPD1707	as JC7623 but <i>lacZ::[recA' ϕluxCDABE cat]</i>
DPD1708	as JC7623 but <i>lacZ::[recA' ϕluxCDABE cat]</i>
DPD1715	as FD1062 but <i>lacZ::[recA' ϕluxCDABE cat]</i>
DPD1716	as CU847 but <i>lacZ::[recA' ϕluxCDABE cat]</i>
DPD1719	as DPD1693 but <i>lacZ::[recA' ϕluxCDABE cat]</i>
DPD1728	as DPD1010 but <i>lacZ::[recA' ϕluxCDABE cat]</i>
DPD1729	as DPD1011 but <i>lacZ::[recA' ϕluxCDABE cat]</i>
DPD1730	as DPD1682 but <i>lacZ::[recA' ϕluxCDABE cat]</i>
JC7623	<i>thr-1 ara-14 leuB6 d(gpt-proA)62 lacY1 tsx-33 supE44 galK2 l- rac- hisG4 (Oc) rfbD1 mgl-51 rpsL31 kdgK51 xyl-5 mtl-1 argE3 (oc) thi-1 qsr' sbcC201 sbcB15 recB21 recC22</i> [from F. Valle, Insitute de Biotechnologia, UNAM, Cuernavaca, Mexico]
DM800	<i>F-</i> , <i>metA28, lacY1 or Z4, thi-1, xyl-5 or -7, galK2, tsx-6</i> [from Brooks Low, Yale University, fully described in <i>J. Bacteriol.</i> , 12:886, (1972)]
DM803	as DM800 but <i>lexA1</i> [from Brooks Low, Yale University, fully described in <i>J. Bacteriol.</i> , 12:886, (1972)]
DPD1714	as DM800 but <i>lacZ::[recA' ϕluxCDABE cat]</i>
DPD1709	as DM800 but <i>lacZ::[recA' ϕluxCDABE cat]</i>

Construction of libraries

Chromosomal DNA isolated from *E. coli* W3110 [B. Bachmann, in *E. coli and Salmonella typhimurium; Cellular and Molecular Biology* (Niedhardt et al., Eds., pp 1190-1220, American Society of Microbiology, Washington, D.C. (1987))] was partially digested with restriction enzyme *Sau3A1* and size fractionated on agarose gels. Fractions of two size ranges (average sizes of approximately 2.5 and 4.0 Kbp) were ligated to pBR322 (0.11 pmol) or pUC18 (0.11 pmol) that had previously been digested with restriction enzyme *Bam*HI and treated with calf intestinal alkaline phosphatase. The molar ratio of chromosomal DNA to vector in each of the ligation reactions was approximately 0.2:1. The ligation products were used to transform ultracompetent *E. coli* XL2Blue (Stratagene) to AmpR. Pooled transformants ($>10^5$ for each transformation) were used to isolate plasmid DNA.

EXAMPLE 115 CONSTRUCTION OF DETECTOR CELL STRAINSConstruction of stain DPD1675

Construction of strain DPD1675 containing *tolC* and *ilvB* proceeded by the manipulation of CU847, an *E. coli* strain, possessing the *ilvB* mutation and having the genotype [*ilvB2101 ara thi Δpro lac*] from H.E. Umbarger, Purdue University. A P1_{vir} phage stock was grown on strain DE112 [*tolC::miniTn10*; fully described in Van Dyk et al., *Applied and Environ. Microbiol.* 60:1414-1420 (1994), isogenic with RM443 described in B. Bachmann, in *E. coli and Salmonella typhimurium; Cellular and Molecular Biology* (Niedhardt et al., Eds., pp 1190-1220, American Society of Microbiology, Washington, D.C. (1987))] by standard methods as described in J. H. Miller, *Experiments in Molecular Genetics*, (1972) Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp 201-205. The phage stock was mixed as described by Miller (*supra*) with strain CU847, tetracycline resistant recombinants were selected and a recombinant that was unable to grow on MaConkey agar (Miller, *supra*) and hence displaying a bile salt sensitive phenotype was designated DPD1675.

Construction of DPD1707

DPD1707 containing the *recA-LuxCDABE* gene fusion was constructed as follows.

Plasmid *precALux3* was isolated from strain DPD2794 (fully described in U.S. 5683868), isogenic with RM443 described in B. Bachmann, in *E. coli and Salmonella typhimurium; Cellular and Molecular Biology* (Niedhardt et al. Eds., pp 1190-1220, American Society of Microbiology, Washington, D.C. (1987)]. The promoter region (*recA*) was amplified by PCR using primers 1,2 (SEQ ID NOS:1 and 2 respectively).

Primer 1:

5' ACT Ttt aagctt AGA GAA GCC TGT CGG CAC 3' recA-upperH (SEQ ID NO:1)

5 Primer 2:

5' AGC TTT ggatecc CGC TTT CTG TTT GTT TT 3' recA-lowerB (SEQ ID NO:2)

The resulting product was digested with *Bam*H1 and *Sal*1 and was mixed with a similarly digested pJT205 plasmid (formerly called pCGLS205) containing the *Photorhabdus luminescens luxCDABE* gene complex, fully described by (Rosson, R. A., PCT International Application WO 93/03179 (1993)). After ligation the mixture was transformed into strain DH5 (ATCC) and ampicillin resistant colonies were selected. The colonies were screened for bioluminescence. One such bioluminescent transformant was designated DPD1657. Plasmid pRecALxx1, isolated from strain DPD1657, was digested with *Pst*1 and *Eco*r1. This digested plasmid was mixed with similarly digested pBrint.CM. plasmid, [from F. Valle, Insituto de Biotechnologia, UNAM, Cuernavaca, Mexico; Balabas et al., *Gene* 172:65-69 (1996)]. After ligation chloramphenacol resistant transformants were recovered in strain DH5. The resistant colonies were screened for a bioluminescent phenotype. One such transformant was termed DPD1696. The plasmid in this strain was called pDEW14. Plasmid pDEW14 contained a fusion of the *recA* promoter to the *Photorhabdus luminescens luxCDABE* gene complex. This fusion is situated within *lacZ* coding sequences. Plasmid pDEW14 was isolated from DPD1696 and the DNA was introduced into strain JC7623 [B. Bachmann, in *E.coli and Salmonella typhimurium: Cellular and Molecular Biology* (Niedhardt et al. Eds., pp 2466, American Society of Microbiology, Washington, D.C. (1987))] by transformation and chloramphenacol resistant colonies were selected. Colonies that were ampicillin sensitive and *lacZ* negative (i.e., could not cleave X-gal) designated as DPD1707. It had the *recALux* fusion integrated into the *lacZ* locus of the *E. coli* chromosome and was bioluminescent.

A phage stock of P1_{vir} was prepared on strain DPD1707 (P1_{vir} and the method are fully described in J. H. Miller, *Experiments in Molecular Genetics*, (1972) Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp 201-205.

35 Construction of DPD1718 (*lacZ::recA'φ'luxCDABE*) proceeded from the strain DPD1692. A P1_{vir} phage stock grown on strain DPD1707 was mixed as described by Miller (*supra*) with strain DPD1692; chloramphenicol resistant recombinants were selected. One such recombinant that also displayed bioluminescence was designated DPD1718.

The same transductional methods were used to create strains DPD1715, DPD1730, DPD1728, DPD1729, DPD1716, DPD1708, DPD1719, DPD1709, and DPD1714's reported in Table 1.

EXAMPLE 2

5 ENHANCED BIOLUMINESCENCE IN THE PRESENCE OF THE DNA DAMAGING AGENT MITOMYCIN C

To test the detector cells for sensitivity to genotoxic substances, strains DPD1715 and DPD1730 were grown to mid-logarithmic phase in LB medium. Strain DPD1715 and DPD1730 differ only in that DPD1730 is *tolC* while
 10 DPD1715 is *tolC*⁺. To the medium was added varying concentrations of Mitomycin C, ranging from 0 to 20 µg/mL. The kinetics of light emission after introduction of the genotoxin was monitored using a microtiter plate format luminometer as described previously (Van Dyk et al., *Applied and Environmental Microbiology* 60, 1414, (1994)) except that the temperature was controlled at
 15 37 °C.

The kinetics are presented in Figures 4 (a) and (b), while a dose-response curve is shown in Figure 5. For DPD1730 [*tolC*⁻] (figure 4(b)), high concentrations of mitomycin C, (here above 2.5 µg/mL) will either inhibit or prevent the increase in light production attributable to growth. At low
 20 concentrations (below 2.5 µg/mL) the cell does not acknowledge the presence of the chemical. At intermediate concentrations (0.63 and 0.31 µg/mL) the cell responds to the damage by inducing the SOS response as conveniently monitored by the large increase in bioluminescence, above the mock treated control, that develops as a function of time. In contrast, the *tolC*⁺ strain, DPD1715 (figure
 25 4(a)) was much less sensitive to mitomycin C (see figure 5). Induction was observed throughout the entire range of mitomycin C concentrations. Inhibitory effects were not observed.

As expected, induction is *lexA*-dependent as indicated by kinetic and dose-response curves of the fusion introduced by transduction into an isogenic pair of
 30 strains differing in the *lexA* gene that controls the SOS response to DNA damage. The *LexA* repressor encoded by *lexA* must be cleaved if the SOS response is to be activated [Walker, 1996, in *E. coli* and *Salmonella typhimurium*; Cellular and Molecular Biology (Niedhardt et al. Eds., pp 1400-1416, American Society of Microbiology, Washington, D.C. (1996))] The *lacZ::recA' φ'luxCDABE* fusion
 35 was introduced by transduction into strains DM800(*lexA*⁺) and DM803 (non inducible *lexA* due to a non-cleavable repressor gene product [Mount et al., *J. Bact.*, 112: 886, (1972)] using a phage stock grown on strain DPD1707. One chloramphenicol transductant from each cross that was bioluminescent was saved and designated as DPD1714 (*lacZ::recA' φ'luxCDABE lexA*⁺) and DPD1709

(*lacZ::recA' ϕ' luxCDABE lexA*). Mitomycin C treatment caused enhanced light production from strain DPD1714 but not from strain DPD1709 indicating that the treatment elicited a true SOS response since the inability to cleave the LexA repressor prevented the bioluminescent increase.

5

EXAMPLE 3

DECREASED BIOLUMINESCENCE AFTER TREATMENT WITH 4 CROP PROTECTION CHEMICALS

Example 3 illustrates the effect of four different crop protection chemicals on the light output of the detector cells.

10

The detector strains were growth to mid-log arithmetic phase as described above treated with sulfometuron methyl, methyl viologen, glyphosate and 2,4-dichlorophenoxyacetic acid (2,4-D) according to the following conditions where all cultures were maintained at 37 °C and tests were run over a time period of 0-90 min.

15

Strain DPD1718 was exposed to 2,4-D over a concentration range of 78-5000 µg/mL. Modulation in bioluminescence is seen in Figure 6.

Strain DPD1715 was exposed to glyphosate over a concentration range of 2.5-2500 µg/mL. Modulation in bioluminescence is seen in Figures 8(a) and (b).

20

Strain DPD1718 was exposed to sulfometuron methyl over a concentration range of 24-1800 µg/mL. Modulation in bioluminescence is seen in Figure 7.

Strain DPD1730 was exposed to methyl viologen over a concentration range of 0.75-48 µg/mL. Modulation in bioluminescence is seen in Figure 9.

25

As is evident from the figures illustrating kinetic data, all crop protection chemicals gave a dose-dependent decrease in light emission from the detector strains.

EXAMPLE 4

tolC ENHANCES THE RESPONSE TO SULFOMETURON METHYL AND MITOMYCIN C

Example 4 illustrates the effect of the *tolC* mutation on the detector cell response to sulfometuron methyl and Mitomycin C. Detector cells were growth to mid-logarithmic phase as described above. Detector cells were exposed to a range of crop protection chemical and bioluminescence was measure at 80 min post-exposure. Strain DPD1715 (*tolC*+) and strain DPD1728(*tolC*) were exposed to Sulfometuron methyl over a concentration range of 0.006 to 0.4 µg/mL and responses are compared in Figures 10(a-c). Similarly *tolC*+ and *tolC* strains were exposed to concentrations of 0-20 µg/mL of mitomycin C and responses are compared in Figure 5. From the data it is clear that the *tolC* mutation has the effect of enhancing sensitivity of the detector strain to the CPC.

The bioluminescent assay demonstrating the efficacy of the *tolC*- strains was confirmed using disk diffusion assays [Stephens et al., *PNAS* 72, 4389, (1975); LaRossa R.A. and J.V. Schloss, *J.Biol.Chem.*, 259, 8753 (1984)] (data not shown).

5

EXAMPLE 5

INDICATIONS OF SITE OF ACTION OF OXIME CARBAMATE OC, GLYPHOSATE AND THIENYLALANINE BY NUTRITIONAL REVERSAL

Example 5 demonstrates the use of a detector cell having to identify the site of action of the oxime carbamate OC, glyphosate and thienylalanine.

10

Strain DPD1718 was grown in minimal medium or LB medium at 37 °C and was exposed to either 3-(2-thienyl)-L-alanine or the oxime carbamate fungicide compound OC over a variety of concentrations. Concentrations of 3-(2-thienyl)-L-alanine ranged between 0 and 100 µg/mL for growth in both minimal and LB medium.

15

Concentrations of compound OC ranged from 0 to 100 µg/mL for cells grown in minimal medium and from 0 to 1000 µg/mL for cells grown in LB medium. Concentrations of glyphosate for cells grown in both minimal and LB Rich medium ranged between 0 and 5000 µg/mL.

20

As seen by the data in Figures 11(a), (b), 12(a), (b) and 13(a), (b), treatment of the detector cell with these compounds caused a decrease in light production in minimal medium, whereas in rich LB medium, light emission was not decreased.

25

Auxanography was used to determine the nutrient responsible for obviating the light-inhibiting effect of these experimental crop protection chemicals.

Auxanographic protocol:

30

Strain DPD1718 was grown to mid-logarithmic phase in minimal medium. A total of 11 individual pools are created which represent various bases, vitamins and amino acids (Tables 2, 5). Each pool shares one component with one other pool. Each pool was added singly into a microtiter well in duplicate.

35

Composition of the pools makes metabolic sense as described in Davis, R. W., D. Botstein And J. R. Roth, *A Manual For Genetic Engineering: Advanced Bacterial Genetics*. X+254p. Cold Spring Harbor Laboratory: Cold Spring Harbor, N.Y., U.S.A., 1980, see pp. 206-208. Minor changes to the described 11 pools from that of Davis et al. were made to accommodate metabolic differences between *Salmonella typhimurium* and *E. coli*. The pool components follow; their concentrations in the pools are as described by Davis et al., *supra*:

pool-components

- 1-adenosine, histidine, phenylalanine, glutamine, thymine
- 2-guanosine, leucine, tyrosine, asparagine, serine
- 3-cysteine, isoleucine, tryptophan, uracil, glutamate
- 5 4-methionine, lysine, threonine, aspartate, DAP
- 5- isoleucine, valine, proline, arginine, glycine
- 6-adenosine, guanosine, cysteine, methionine, isoleucine
- 7-histidine, leucine, isoleucine, lysine, valine
- 8-phenylalanine, tyrosine, tryptophan, threonine, proline, PABA, DHBA
- 10 9-glutamine, asparagine, uracil, aspartate, arginine
- 10-thymine, serine, glutamate, DAP, glycine
- 11-pyridoxine, nicotinate, biotin, pantothenate, alanine

The volume added per well was 10 μ L of an individual pool plus 40 μ L of fresh minimal media which has been supplemented with a predetermined inhibitory concentration of a given compound. Fifty microliters of midlog arithmic cells grown in minimal media were added to the microtiter well. The microtiter plate was then placed into a ML2000 luminometer and the bioluminescence of the sample was monitored over time. The data was compared to the sample of cells not challenged with the compound. The pool that enabled the cell to overcome the inhibitory effects of the compound, as determined by a restoration in bioluminescence, was determined to be the pool that contained the component that reversed the dampening effect of the compound. Finally, individual components of the pool were tested to determine the specific ingredient responsible for restoration of bioluminescence.

25 Nutritional Reversal of Compound OC Action

Table 2 records the data from the interaction of the compound OC with DPD1718. A lights-off response, (approximately 1000 fold diminution in light output) is observed on minimal but not rich media. Auxanography (Table 3) indicated that the presence of pool 6, containing cysteine, was able to prevent the lights-off response.

TABLE 2

Cycle 10:

Cmpd OC	ADDITION	%LUM
-	-	=100
+	Pool 1	0.051
+	Pool 2	0.13
+	Pool 3	0.051
+	Pool 4	0.042
+	Pool 5	0.022
+	Pool 6	89
+	Pool 7	0.070
+	Pool 8	0.095
+	Pool 9	0.028
+	Pool 10	0.11
+	Pool 11	0.13
+	-	0.10

* Medium: E+BI+glucose+glycine+proline+uracil 37 °C/DPD1718/compound OC at 25 µg/mL

A weak prevention of the light-off response was seen at some time points with pool 3 (data not shown). Thus the individual components of pools 3 and 6 were tested for their ability to prevent the lights off response:

5

TABLE 3

Cmpd. OC	ADDITION	%LUM
Pool 6 components		
-	-	=100
+	Pool 6	90
+	Ade	0.053
+	Gua	0.038
+	Cys	94
+	Met	0.034
+	Ile	0.027
+	-	0.031

Cmpd. OC	ADDITION	%LUM
Pool 3 components		
+	Pool 3	0.048
+	Cys	94
+	Ile	0.027
+	Trp	0.046
+	Glut	0.13
+	Ura	0.027

It is evident that the reversing agent is cysteine, the common component between the 2 pools. Cysteine could exert its effects either by reversing a chemical-induced auxotrophy or by forming an adduct with the oxime carbamate or with its hydrolysis product. To attempt to distinguish between these 2 models, the stereoisomers of cysteine were tested as reversing agents:

TABLE 4

Supplement	%LUM		
	Inhibitor:		
	Cmpd. OC	Analog	None
-	.04	.06	=100
L-cys	45	45	
D-cys	69	75	

10 It is apparent that D-cysteine can also reverse compound OC effects. Applicants then tested and found that D-cysteine satisfies the auxotrophy of 2 *cysD* strains, a *cysA* mutant, a *cysH* strain and a *cysB* mutant. The data suggests that either the D-cysteine is not optically pure or that *E. coli* has the ability to interconvert the two stereoisomers.

15 Additional experiments, showed that a free sulfhydryl group, present on a variety of compounds including 2-mercaptoethanol, was responsible for quenching the profungicide inhibition of bioluminescence (data not shown). The finding indicated that compounds with free SH groups obviate compound OC action, suggesting that L-cysteine biosynthesis is not compromised by compound
20 OC action. Thus, nutritional reversal experiments can suggest agents capable of obviating inhibitors action. Such experimentation must be buttressed with further investigation.

Nutritional Reversal of 2-Thienylalanine Action

Nutritional reversal of thienylalanine inhibition was accomplished in a similar fashion as with compound OC. Pools were created as described above. Reversal of thienylalanine inhibition by pools 1 and 8 was observed as shown in Figure 14. The reversal can be attributed to phenylalanine, but not to the other components of the pools.

Nutritional Reversal of Glyphosate Action

Nutritional reversal of glyphosate inhibition was accomplished in a similar fashion as with compound OC and thienylalanine. Reversal of glyphosate inhibition only by pool 8 was observed as shown in Figure 13(a-b) and Table 5.

TABLE 5

Cycle 10:

Glyphosate	ADDITION	%LUM
-	-	=100
+	Pool 1	19
+	Pool 2	19
+	Pool 3	16
+	Pool 4	18
+	Pool 5	29
+	Pool 6	14
+	Pool 7	23
+	Pool 8	105
+	Pool 9	19
+	Pool 10	19
+	Pool 11	19
+	-	18

*Medium: E+B1+glucose+glycine+proline+uracil 37 °C/DPD1718/Glyphosate at 600 µg/mL

Reversal by pool 8 only is indicative of a block in the common aromatic amino acid pathway (Davis et al., *supra*, p. 208). Thus the target pathway of glyphosate is accurately identified by this method.

Nutritional Reversal of Sulfometuron Methyl (SM) Action

The bioluminescence of Strain DPD1718 growing in minimal E medium supplemented with thiamine, glucose and uracil was inhibited by sulfometuron methyl in a dose-dependent manner (see Figure 15(a) in which the concentrations tested were 1800, 900, 450, 225, 113, 61, 31 and 0 µg/mL).

Nutritional reversal of SM inhibition caused by administration of 250 µg/mL of the herbicide is depicted in the bottom panel of Figure 15b. The most effective pool at reversing the inhibition was pool 7 composed of the three branched chain amino acids, lysine and histidine. Reversal by this pool is consistent with the known site of SM action. Incomplete reversal to a lesser degree and with greatly delayed kinetics was seen with pools 1 (adenosine, histidine, phenylalanine, glutamine, thymine), 9 (glutamine, asparagine, uracil, aspartate, arginine) and 11 (pyridoxine, nicotinate, biotin, pantothenate, alanine).

EXAMPLE 6

10 IDENTIFICATION OF SITE OF ACTION OF SULFOMETURON METHYL AND GLYPHOSATE BY GENETIC TITRATION

Characterization of GP and SM Sensitivity by Analysis of Their Interactions with Selected Mutants

Glyphosate is expected to be inhibitory to *E. coli* K12. In contrast, sulfometuron methyl is not inhibitory towards *E. coli* due to the presence of the refractile ALS I isozyme encoded by *ilvBN*. Both compounds inhibit an *ilvB* mutant of *E. coli*. Zone of inhibition assays demonstrate that introduction of a *relA* null allele which prevents the mounting of the stringent response to amino acid starvation into the *ilvB* mutant results in hypersensitivity to GP but does not change the response to sulfometuron methyl.

SM resistant meromultiploids

Strain DPD1675 expresses a single SM-sensitive ALS, ALS III, encoded by *ilvIH* due to the presence of an *ilvB* mutation that prevents production of the wild type ALS I naturally resistant to SM. It also contains a *tolC* mutation that prevents an efflux pump mediated expulsion of SM from the cytoplasm. These two mutations together create a strain quite sensitive to SM. Applicants determined the MIC of strain DPD1675 to be 3 µg/mL in minimal medium containing plates.

The genetic titration of the herbicide sulfometuron methyl was performed in the strain DPD1675 (Table 1). This strain harbors a mutation in the *ilvB* allele and *tolC*, which result in rendering the cell sensitive to the herbicide and more permeable to hydrophobic compounds. The MIC of the herbicide in this strain was determined to be 3 µg/mL following 1 day of growth on minimal plates.

Frozen competent cells of DPD1675 (prepared by the method of Nishimura et al., *Nucleic Acids Research* 18:6169 (1990)) were transformed with 0.5 µL of plasmid DNA from two different *E. coli* libraries, one pBR322-based and one pUC18-based. The transformation mix was washed one time and resuspended in 1 X E [Davis et al., *supra*, pp. 202-203] before being plated to minimal E plates supplemented with thiamine, proline, glycine and glucose at

standard concentrations that can be found in Davis et al., *supra*, pp. 201-210, and Miller, 1972, *supra*, p. 432. The selection for the desired clones was Ampicillin (100 µg/mL) and Sulfometuron methyl (9 µg/mL) which was also included in the media.

- 5 Strain DPD1675 was transformed with 2 libraries containing random fragments of the *E. coli* chromosome ligated into either pBR322 or pUC18. Plasmids from 19 isolates, when reintroduced into strain DPD1675, conferred resistance to SM (Table 6).

TABLE 6

Selection		Plasmid:			
<u>Agent</u>	<u>µg/mL</u>	<u>Host</u>	<u>Vector</u>	<u>Name</u>	<u>Inserted Genes</u>
SM	9	DPD1675	pBR322	pDEW21	<i>tolC</i>
				pDEW18	<i>tolC</i>
				pDEW22	<i>tolC</i>
				pDEW123	<i>tolC</i>
				pDEW24	<i>tolC</i>
				pDEW25	<i>tolC</i>
				pDEW20	' <i>emrDivbL ilvBN uhpA</i> <i>uhpB uhpC uhpT</i> '
				pDEW26	<i>glvA glvB glvG yidL</i> <i>yidK yidJ yidI yudH</i> <i>yidG tidF emrD evbL</i> <i>ilvBN uhpA uhpB uhpC</i> <i>uhpT</i> '
				pDEW27	<i>yidG' yidF emrD ivbL</i> <i>ilvBN uhpA uhpB</i> '
				pDEW28	' <i>yidF emrDivbL ilvBN</i> <i>uhpA uhpB uhpC</i> '
				pDEW19	<i>ilvIH</i>
				pDEW15	' <i>emrDivbL ilvBN uhpA</i> <i>uhpB</i> '
				pDEW34	' <i>yidF emrDivbL ilvB</i> <i>ilvN</i>
SM	9	DPD1675	pUC18	pDEW29	<i>yidG' yidF emrD ivbL</i> <i>ilvBN uhpA uhpB uhpC</i> '
				pDEW30	<i>ilvBN</i> '

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				pDEW31	<i>yidI' yidH yidG yidF</i> <i>emrD ivbL ilvB ilvN</i>
				pDEW32	<i>'emrDivbL ilvBN uhpA</i> <i>uhpB uhpC'</i>
				pDEW17	<i>ilvIH</i>
GP	560	DPD1692	pUC18	pDEW110	<i>'yhhR yhhS yhhT yhhU'</i>
				pDEW112	<i>yhhQ yhhR yhhS yhhT</i> <i>yhhU'</i>
GP	560	DPD1692	pBR322	pDEW114	<i>'f589 o230 serC aroA</i>
				pDEW115	<i>'f307 o552 criR f461</i> <i>ybeG cspE ybeI ybeH'</i>
				pDEW116	<i>'f415 f108 glk o418 o108'</i>

The response of these transformants to bile acids was scored; bile acid sensitivity indicates a *tolC*⁻ phenotype while bile acid resistance indicates a *tolC*⁺ phenotype. The introduction of 6 plasmids into DPD1675 resulted in a bile salt resistant isolate suggesting that these strains are *tolC*⁺/*tolC* heterozygotes and that *tolC*⁺ was dominant. This supposition was confirmed by sequencing both strands of each insert. The primers used for sequencing the inserts into pBR322 were:

10 GCC ACT ATC GAC TAC GCG (SEQ ID NO: 3)

CTG TGG CGC CGG TGA TGC (SEQ ID NO: 4)

while those used for sequencing the inserts into pUC18 were:

15 GTA AAA CGA CGG CCA GT (SEQ ID NO: 5)

AGC GGA TAA CAA TTT CAC ACA GGA (SEQ ID NO: 6).

20 Informatics methods were used to place the obtained sequences onto the *E. coli* genome (Genbank Accession number U00096). These data (Table 6) led Applicants to conclude that each plasmid conferring both SM and bile salt resistance carried *tolC*⁺.

25 Strain DPD1675 containing the other 13 plasmids maintained the bile salt sensitive phenotype of the plasmid free host. The nineteen plasmids were also used to transform strain MF2000. MF2000 harbors mutant *ilvBN* and *ilvIH* alleles and so is devoid of ALS activity. As such, the strain is an isoleucine-valine auxotroph. Introduction of the *tolC*⁺ containing plasmids does not alter the observed auxotrophy of strain MF2000. In contrast, the transformants of strain

MF2000 containing the other 19 plasmids were isoleucine-valine prototrophs. This indicated that they were expressing either ALS I or ALS III and that the plasmids were likely to harbor *ilvBN* or *ilvIH*. These inserts' ends were also sequenced leading to the identification of the genes harbored on the inserts by comparison with the complete primary sequence of the *E. coli* genome (Genbank Accession number U00096; see Table 6). From the data presented we concluded that there were 9 *ilvBN*⁺/*ilvBN* heterozygotes (see Table 6) and 2 *ilvIH*⁺/*ilvIH*⁺ homozygotes in the original collection of SM-resistant meromultiploids.

Selection and characterization of glyphosate resistant meromultiploids

Strain DPD1692 was used for the selection of glyphosate resistant meromultiploids. Applicants determined the MIC of strain DPD1692 to be 3.0 mM (0.56 mg/mL) glyphosate in minimal media plates.

Competent DPD1692 cells transformed with 0.5 uL (of either library one (pBR22-based; 0.0375 µg DNA) or library three (pUC18-based, 0.15 µg, DNA) yielded 10⁴ transformants thus having a frequency of about 3 x 10⁵ and 0.7 x 10⁵ transformants per microgram DNA, respectively when plated on rich media plus ampicillin. Approximately 2 x 10³ cells were plated per selective plate which consisted of minimal E media amended with glucose, glycine, proline, uracil and thiamine as well as the selective agents Ampicillin (100 µg/mL) and glyphosate (3.3 mM = 0.56 mg/mL). Glyphosate resistant meromultiploids appeared throughout a 96 h incubation at 37 °C.

Glyphosate selection resulted in the isolation of 7 transformants from the pBR#22 library, and 18 transformants from the puC18 library (Table 6). Retransformation of the original host strain DPD1692 with the 25 plasmids conferred glyphosate resistance, insuring that the plasmid conferred the resistance phenotype. Presence of *aroA*, the gene that encodes the known target of glyphosate, was screened by transforming an auxotrophic *aroA* mutant *E. coli* strain AB2829 with the plasmids that conferred glyphosate resistance. Prototrophic transformants indicated that the *aroA* gene was resident on the glyphosate-resistance conferring plasmid. Two of the 25 isolated plasmids restored growth of AB2829 on minimal media. The presence of *aroA* on these two plasmids was confirmed by sequence analysis (see Table 6) using the primers described above. These two plasmids conferred the strongest resistance to glyphosate when the 25 glyphosate resistance plasmids were compared in strain DPD1692 (data not shown).

Of the remaining 23, non-*aroA* containing plasmids, three distinct inserts conferring novel glyphosate resistances have been identified by sequence analysis (See Table 6). Group A had 17 members, group B had 4 members and group C had 2 members. Localization of the inserts relative to the *E. coli* genome map was

accomplished by FASTA and BLAST (Wisconsin Package Version 9.0, Genetics Computer Group (GCG), Madison, Wisc.) scans of the databases. The group A inserts, mapping to 77.8 minutes on the *E. coli* chromosome, contain two previously sequenced ORF's, *yhhS* and *yhhT*. The predicted protein structures for both of these ORF's contain domains predicted to span the membrane and they are thus likely to be permeases. The 4 members of the second group, B, mapping to the 14.2 minute region, contain an uncharacterized region of the *E. coli* chromosome. The third group of two inserts maps to the 52.3 minute region of the *E. coli* chromosome. Sequence analysis has revealed genes related to carbohydrate metabolism are contained in this insert. ORF's identified to date include previously undescribed component IIC and IIB PTS enzymes. The predicted amino acid sequence encoded by these two genes share homology with component IIC and IIB enzymes encoded by a fructose-like PTS operon (Reizer, J. A. et al., *Microbiol.* 141, 961, (1995)). In addition to new PTS enzyme II homologues, this insert contains the single *E. coli* glucokinase gene, *glk*.

EXAMPLE 7

IDENTIFICATION OF MITOMYCIN C - RESISTANCE GENES

Example 7 illustrates the identification and isolation of genes having resistance to Mitomycin C that may be used in the construction of a detector cell containing the *recA-LUX* fusion.

Genetic Titrations of Mitomycin C Action

Mitomycin C (a DNA damaging agent) inhibits colony formation of a *lexA^{ind}* mutant of *E. coli*, DM803, with a MIC of 3 µg/ml. This MIC is 5 x lower than that of the *lexA⁺* strain DM800. Competent DM 803 cells were transformed with a pBR322 library and pUC18 library. Colonies were selected by plating on LB Amp 150 plates in the presence of 15µg/ml of mitomycin C (3X the MIC) at 37°C. After 2 days, 4 colonies appeared on plates where cells were transformed with the pUC18 based *E. coli* library. These 4 colonies were picked; all plates were incubated for another 5 days without the appearance of further colonies. Plasmids were purified from the four colonies and named plexA3.1, 3.2, 3.3 and 3.4. Reintroduction of each plasmid by retransformation of strain DM803 showed linkage between the ampicillin resistance and mitomycin C resistance indicating that the mitomycin C resistance in each case was a plasmid encoded trait.

Plasmid purification from DM803 host was difficult - poor yield and degradation of samples upon storage were observed. Thus plasmids were transferred to RFM443 for routine purification of plasmid template for sequencing.

Sequences obtained:

(1) The two ends of the LexA3.1 insert were sequenced. LexA3.1 forward-primed sequence maps to region 291 out of 400 (minute 18) as defined by the Blattner *E. coli* sequencing project - *glt* region, with putative fimbrial chaperone gene, and *yhcA* while the reverse-primed sequence maps to region 76 out of 400 - this region contains *dacC* (a penicillin binding protein) and *deoR* (deoxyribose operon repressor) as well as several open reading frames encoding proteins of unknown function. Since these are two non-continuous regions of the chromosome, the selected plasmid is a chimera of 2 non adjacent fragments presumably fused during the library construction.

(2) LexA3.2 forward- and reverse-primed sequences map to region 76 out of 400 in *E. coli* database. This region contains *dacC* (a penicillin binding protein) and *deoR* (deoxyribose operon repressor) among the 8.6kb found in the insert. The Lex A3.3 reverse-primed sequence: maps to the same region as LexA3.2; while the forward-primed sequence is unavailable at this time.

(3) Similarly the Lex A3.4 reverse-primed sequence also maps to same region as LexA3.2 and appears to contain the same junction as Lex3.3; again forward-primed sequence is unavailable.

Thus it appears that a gene or genes within the region 76 of the chromosome of *E. coli* confers multicopy resistance to mitomycin C. It is obvious that division of the segment and retesting will define a novel gene or genes responsible for mitomycin C resistance.

These genes and open reading frames included on pLexA3.2 are: *o127, o371, f210, dacC* = penicillin-binding protein 6 precursor, *deoR* = deoxyribose operon repressor, *f198, o410=mdfA, f94, f262, f402 & o178*.

Further experiments were performed with a strain proficient in the SOS response since the non-inducible allele could compromise a subset of potential resistance mechanisms. Strain RFM443 is *lexA*⁺ and thus was used for genetic titration with mitomycin C. The MIC for mitomycin C for this strains was determined to be between 1 and 3 µg/ml on rich, solidified LB medium. Thus LB solidified with agar and supplemented with 6 µg/ml of mitomycin C and 150 µg/ml of ampicillin was used to select mitomycin resistant clones after transformation of strain RFM443 with *E. coli* genomic libraries constructed in either pBR322 or pUC18. Plasmid DNA purified from such resistant isolates was reintroduced into RFM443 to demonstrate if the resistance was a plasmid encoded trait. DNA sequencing of the vector-insert junctions served to define those sequences that conferred resistance to mitomycin C. Such resistances mapped to 3 sites as defined by sequencing of the inserts (see following).

- Site A, isolated 7 times, coincides with the site at minute 18 present in the pLexA3 plasmids (above). This set of plasmids, however, (see Table 7 below) demonstrates that one specific gene, *o410* (*mdfA*), a recently described multidrug transporter, in the minute 18 region is capable of conferring resistance to
- 5 mitomycin C when present in multiple copies. The inhibitory action of another DNA damaging agent, C0360, is not effected by site A clones.

TABLE 7
Site A Clones.

10

RESPONSE TO:

clone (name)	vector	insert (intact orfs included)	MMC 15µg/ul backcross	C0360 200 µg/ml
1+2/A4	pBR322	<i>f198,o410,f94,f262</i>	+	--
3+4/B1	pUC18	<i>f198,o410,f94,f262</i>	--	ND
3+4/B7	pUC18	<i>f198,o410,f94</i>	--	ND
1+2/B13	pBR322	<i>deoR,f198,o410</i>	ND	ND
1+2/B14	pBR322	<i>o410,f94,f262</i>	+	--
1+2/B16	pBR322	<i>o410,*</i>	+	--
1+2/B17	pBR322	<i>deoR,f198,o410</i>	+	--

ND-not determined; + indicates growth; - indicates no growth

- Site B mediated resistance, due to high dosage of the minute 43 region, is defined by 16 distinct inserts (see Table 8 below). The only intact gene shared in common
- 15 by these inserts from site B is *sdiA*, which encodes a positive activator of *ftsQAZ* operon whose products are essential for cell division. Both the *sdiA* and *rpoS* gene products act on distinct promoters of *ftsQAZ*. The inhibitory action of the DNA damaging agent C0360 is lessened by the presence of several site B clones.

20

TABLE 8
Site B Clones

RESPONSE TO:

clone (name)	vector	insert (intact ORFs listed)	MMC 15µg/ul backcross	C0360 200 µg/ml
1+2/B3	pBR322	<i>uvrY,*</i>	+	+
1+2/B4	pBR322	<i>uvrY,yecF,sdiA</i>	--	--
1+2/B6	pBR322	<i>yecF,sdiA,f250</i>	+	+
1+2/B7	pBR322	<i>yecF,sdiA,f250</i>	--	+
1+2/B8	pBR322	<i>uvrY,yecF,sdiA</i>	--	--
1+2/B9	pBR322	<i>sdiA,*</i>	--	+

1+2/B10	pBR322	<i>sdiA,f250,yecC,f360</i>	+	+
1+2/B11	pBR322	<i>yecF,sdiA,f250,yecC</i>	+	+
1+2/B12	pBR322	<i>yecC,*</i>	+	--
1+2/B15	pBR322	<i>uvrY,yecF,sdiA,f250</i>	+	+
1+2/B18	pBR322	<i>uvrY,yecF,sdiA</i>	+	+
3+4/B4	pUC18	<i>uvrY,yecF,sdiA</i>	+	ND
3+4/B10	pUC18	<i>yecF,sdiA</i>	+	ND
3+4/B12	pUC18	<i>yecF,sdiA,f250,yecC</i>	+	ND
3+4/B14	pUC18	<i>yecF,sdiA,f250,yecC</i>	+	ND
3+4/B15	pUC18	<i>yecF,sdiA,f250,yecC</i>	+	ND

* only one end of these inserts were sequenced; ND-not determined; + indicates growth;
- indicates no growth

10 Site C inserts were identified by sequencing (see Table 9 below). Arising from
5 the minute 44 region, they have but one common intact gene, *sbmC*. Expression
of this gene is induced by microcin B17, a small peptide antibiotic that causes
double strand DNA breaks, other DNA damaging agents and entry into stationary
phase. A limited sampling indicates that site C clones do not confer cross-
resistance to the DNA damaging agent C0360.

10

TABLE 9
The clones in the *sbmC* region, site C.

RESPONSE TO:				
clone (name)	vector	insert (intact ORFs listed)	MMC 15µg/ul backcross	C0360 200 µg/ml
3+4/B2	pUC18	<i>sbmC</i>	+	ND
3+4/B3,	pUC18	<i>o148,o73,o122,</i>	+	ND
3+4/B5,		<i>o124,o64,f131,</i>	+	ND
3+4/B6,		<i>yeeA,sbmC,yeeC</i>	+	ND
3+4/B9,			+	ND
3+4/B11,		(note: these clones are	+	ND
3+4/B13,		not identical)	+	ND
3+4/A1			+	--
3+4/B8	pUC18	<i>o512,o148,o73,</i> <i>o122,o124,o64,f131,</i> <i>yeeA,sbmC,yeeC</i>	+	ND
3+4/A2	pUC18	<i>sbmC</i>	+	--

ND-not determined; + indicates growth; - indicates no growth

15

EXAMPLE 8IDENTIFICATION OF ACIVICIN RESISTANCE GENES

Example 8 illustrates the identification and isolation of genes having resistance to Acivicin that may be used in the construction of a detector cell containing the *recA-LUX* fusion.

Genetic Titrations of Acivicin Action

Acivicin inhibits colony formation of *E. coli* strain DPD1675 on minimal medium with a MIC of 1 µg/ml.

Competent DPD1675 cells were transformed with a pBR322 library and pUC18 library each containing random fragments of the *E. coli* chromosome. Colonies were selected by plating on E plates supplemented with glucose, thiamine, proline, 100 µg/ml ampicillin and 3 µg/ml of acivicin (3X the MIC) at 37°C. After prolonged incubation, colonies appeared on the plates. Plasmids were purified from the colonies and named. Reintroduction of each plasmid by retransformation of strain DPD1675 showed linkage between the ampicillin resistance and acivicin resistance indicating that the acivicin resistance in each case was a plasmid encoded trait.

Plasmid purification from the resistant clones provided a plasmid template for sequencing.

Sequences obtained

Class 1 clones come from region 287 at about 43 minutes. 9 in number, their inserts vary from about 2800-5800 bp but all contain an intact *yedA*. Perusal of Table 10 indicates that no other gene in this region is intact in all class 5 clones that confer resistance.

There is but a single class 2 clone that maps to region 374 at about 69 minutes. It contains a 1831 bp insert that contains the intact *iciA* and *f76* genes. Relevant clones are identified in Table 10:

TABLE 10

CLONE NAME	TOTAL SIZE	GENES
1C2ii	4820bp	'o271 f569 f60 yedI yedA vsr'
1D3ii	4774bp	'o271 f569 f60 yedI yedA vsr'
3C2	2800bp	'yedI yedA vsr dcm'
L1(3B61)	5340bp	'f569 f60 yedI yedA vsr dcm'
L26(3A49)	5197bp	'o271 f569 f60 yedI yedA vsr'
1A7 (E41)	5072bp	'o271 f569 f60 yedI yedA vsr dcm'
L21(3A43)	5340bp	'f569 f60 yedI yedA vsr dcm'
L51(3D39)	5858bp	'o271 f569 f60 yedI yedA vsr dcm'
L56 (3E54)	3838bp	' f60 yedI yedA vsr dcm'
3C12 (E46)	1831bp	'rpiA f76 iciA sbm'

EXAMPLE 9

IN VIVO INHIBITOR IDENTIFICATION OF
THIENYLALANINE LIKE COMPOUNDS

5

Example 9 illustrates the identification and isolation of genes having resistance to Thienylalanine that may be used in the construction of a detector cell containing the *recA-LUX* fusion. The method proceeded by the isolation of Thienylalanine resistant genes and transformation of an appropriate detector cell.

10 Genetic Titrations of Thienylalanine Action

Thienylalanine inhibits colony formation of *E. coli* strain DPD1675 on minimal medium with a MIC of 75 µg/ml.

Competent DPD1675 cells were transformed with a pBR322 library and pUC18 library each containing random fragments of the *E. coli* chromosome.

15 Colonies were selected by plating on E plates supplemented with glucose, thiamine, proline, 100 µg/ml ampicillin and 150 µg/ml of thienylalanine (2X the MIC) at 37°C. After prolonged incubation, colonies appeared on the plates. Plasmids were purified from the colonies and named. Reintroduction of each plasmid by retransformation of strain DPD1675 showed linkage between the

20 ampicillin resistance and thienylalanine resistance indicating that the thienylalanine resistance in each case was a plasmid encoded trait.

Plasmid purification from the resistant clones provided a plasmid template for sequencing.

Sequences were obtained that yielded the following plasmid insert

25 structures, shown in Table 11:

TABLE 11

strain name	clone	predicted size	E. coli genes on plasmid
DPD1748	1.2.1*****	5544 bp	ydiG(A), aroH, ydiE, f478, f237
	1.2.66	14495 bp	ydiG(A), aroH, ydiE, f478, f237, nlpC, btuDEC, himA, pheT
	1.3.41	12831 bp	o312, o429, o97, ydiD, ppsA, ydiG(A), aroH, ydiE, f478, f237
	1.3.51	12831 bp	o312, o429, o97, ydiD, ppsA, ydiG(A), aroH, ydiE, f478, f237
	1.3.66	12832 bp	o312, o429, o97, ydiD, ppsA, ydiG(A), aroH, ydiE, f478, f237
DPD1749	1.2.6	1820 bp	o245, ygaH
	1.2.11	1828 bp	o245, ygaH
	1.2.56	4196 bp	proX, o88, o305, o245, ygaH
DPD1750	1.3.1*****	4356 bp	proX, o88, o305, o245, ygaH
	1.3.6	4188 bp	proX, o88, o305, o245, ygaH
	1.3.11	4186 bp	proX, o88, o305, o245, ygaH
	1.3.26	4139 bp	proX, o88, o305, o245, ygaH
	1.3.31	4188 bp	proX, o88, o305, o245, ygaH
	1.3.36	4188 bp	proX, o88, o305, o245, ygaH
	1.3.46	4188 bp	proX, o88, o305, o245, ygaH
	1.3.56	4188 bp	proX, o88, o305, o245, ygaH
	1.3.71	4188 bp	proX, o88, o305, o245, ygaH

Introduction of plasmid pAHH1 (a multicopy plasmid obtained from R. Baurele,
 5 Univeristy of Virginia, that contains *aroH* but not adjacent genes) into strains
 DPD1675 results in a thienylalanine resistant phenotype indicating that *aroH* is
 the gene responsible for the multicopy mediated resistance.

The clones isolated by thienylalanine resistance were of two classes. One
 resistant class defined by overlapping regions of 12 independently isolated clones
 10 contained the genes *o245 yagaH* which are located at 59.79 minutes of the *E. coli*
 chromosome. The other resistant class defined by overlapping regions of 5
 independently isolated clones contained a region located at 38.22 minutes on the
E. coli chromosome including *ydiG(A) aroH ydiE f478*.

Subcloning the individual ORFs from DPD1750 (*o245 yagaH*)

15 The strain DPD1750 contains the largest insert containing *proX o88 o305*
o245 ygaH (pDEW45). PCR amplification utilizing plasmid DNA isolated from
 DPD1750 (pDEW45) was used as template to subclone either the *o245* or *ygaH*

ORF independently. The primers were designed with defined restriction sites flanking the ORF to allow one to PCR amplify each individual ORF and then directionally clone into pBR322. The forward direction primers were constructed such that they contain a *Bam*HI restriction site (5' GGA TCC 3') incorporated into their nucleotide sequence. The reverse primers have an *Eco*RI restriction site (5' GAA TTC 3') incorporated into their nucleotide sequence. The specific primers for the PCR reaction to isolate the *o245* gene were "o245f" and "o245r", respectively. The specific primers for the PCR reaction to isolate the *ygaH* gene were "ygaHf" and "ygaHr", respectively. Their specific nucleotide sequences are described in below.

"o245f" 5' GTT AGC GGA TCC CTA ATT TCA GCC TCA GCC3' [SEQ ID NO: 7]

"o245r" 5'AGC AGT GAA TTC GTG TAT CGT GCA TCA CTT C3' [SEQ ID NO:8]

15 "ygaHf" 5'GTT AGC GGA TCC TAA TAG GGG CAT TCT CCG3' [SEQ ID NO:9]

"ygaHr" 5'AGC AGT GAA TTC CAG CCG ATA TAG TAA CGA CAG3' [SEQ ID NO:11]

The PCR reaction was run for 40 cycles of 94°C, 1 min.; 50°C, 1 min.; 72°C, 1 min. and the primer conditions were 100 pmole each. The concentrations and sizes of the PCR products were confirmed by electrophoresis on a 2.0% agarose gel. The *o245* PCR reaction yielded a product of the predicted 1236 bp. Likewise, the *ygaH* PCR reaction amplified a product of the predicted size 661 bp.

The PCR products were purified by column filtration (Microcon) and then enzyme digested. Sequential *Bam*HI and *Eco*RI restriction digestions were performed on the *o245* and *ygaH* PCR products and pBR322 vector which would serve as the host vector during the ligation. A fraction of the samples were run on a 0.7% agarose gel to determine their DNA concentrations. Ligation reactions were performed over night at 4°C into the host vector pBR322 with either the *ygaH* or *o245* digested PCR products as the insert DNA. Aliquots of the ligated pDNA was transformed into the DH5α host selecting ampicillin resistance at 150 µg/ml and screening for tetracycline sensitivity at 20 µg/ml. Plasmid DNA was isolated from the ampicillin resistant, tetracycline sensitive isolates.

Transformations into DPD1718 for Reverse Genetic Titrations

The *o245* ligated plasmid (pDEW40) or the *ygaH* ligated plasmid(pDEW39), were transformed into DPD1718 selecting ampicillin 150 µg/ml resistance and screening for bioluminescence, chloramphenicol (20 µg/ml) and kanamycin resistance (25 µg/ml). The host strain DPD1718 contains a chromosomally integrated *recA::lux P. l.*, making the basal level of bioluminescence very high in terms of RLU's (relative light units). Other plasmids were transformed into DPD1718 to serve as positive and negative

controls, namely pBR322, AH1 (*aroH*) (isolated from CB18), and *ppheA*, isolated by selecting for a pBR322 clone that complements a *pheA* auxotroph.

Experimental Protocol

Strains were grown at 37°C in minimal E medium supplemented with 0.4% thiamine and 0.4% glucose with shaking. The cultures were diluted into fresh minimal media the next morning and grown to early log. Serial two fold dilutions of a thienylalanine stock solution (10 mg/ml dissolved in dH₂O) were performed into minimal media in a microtiter plate with highest concentration @ 100 µg/ml. The bioluminescence was monitored for one hour and the kinetic data collected.

The plotted data is shown in figure 16 as ratios (normalized to the unchallenged cells in terms of light production). Only the last time point is shown at all of the concentrations (usually at approximately 60 minutes).

Zone assays were also performed on the cells according to the following protocol. Strains were grown overnight at 37°C in LB medium supplemented with 150 µg/ml of ampicillin. Cultures were collected by centrifugation prior to resuspension in an equal volume of E medium. 0.1 ml portions were plated in 2.5 ml of E medium amended with 0.7% agar to effect an even lawn of cells on E agar plates supplemented with glucose, proline, uracil and 100 µg/ml ampicillin. Filter disks containing the indicated quantities of compounds were placed upon the lawns. Plates were incubated overnight at 37°C before zones of clearing were measured.

Zone assays confirmed the trend in sensitivity to thienylalanine as illustrated in Table 12.

TABLE 12

zone of inhibition (dia. in mm)

compound	µg/disk	DPD1749 (multicopy <i>ygaH o245</i>)	DPD3134 (vector alone)
D,L-allyl glycine	200	24t	26c
1,2,4-triazole	200	15t	17c
5-methyl-D,L-tryptophan	200	15t, 23vt	14c, 24t
D,L-5-aza-tryptophane hydrate	200	30c, 41t	38c
Beta-2-thiazolyl-D,L-alanine	200	nz	15c, 22t
mimosine	200	11c	15c
azaleucine	200	14t	23c, 43t
thienylalanine	200	nz	60c
sulfometuron methyl	40	40c	42c

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glyphosate	200	18c, 25t	27c
rifampicin	1000	35c	40c
nz=nozone			

Relevant genotypes of the strains employed are given in Table 13.

TABLE 13

pDEW40	<i>o245</i> insert in pBR322-PCR amplified from DPD1750
pDEW 39	<i>ygaH.3</i> insert in pBR322- PCR amplified from DPD1750
pDEW45	<i>proX o88 o305 0245 ygaH</i> in pBR322 thienylalanine resistant clone
DPD1748	<i>ydiG(A) aroH ydiE f478 f237</i> in pBR322 thienylalanine resistant clone
DPD1749	<i>0245 ygaH</i> in pBR322 thienylalanine resistant clone
DPD1750	<i>proX o88 o305 0245 ygaH</i> in pBR322 thienylalanine resistant clone
DPD1770	pDEW 39/DH5 α
DPD1771	pDEW 39/ DH5 α -second transformant(<i>ygaH.5</i>) of pDEW39
DPD1772	pDEW40/ DH5 α
DPD1773	pDEW 39/ DPD1675
DPD1774	pDEW 39/ DPD1675 -a second transformant (<i>ygaH.5</i>) of pDEW39
DPD1775	pDEW40/DPD1675
DPD1777	pDEW 39/DPD1718
DPD1778	pDEW 39/ DPD1718-a second transformant (<i>ygaH.5</i>) of pDEW39
DPD1779	pDEW40/DPD1718
DPD1780	pDEW45 /DPD1675
DPD1675	<i>ilvB tolC</i>
DPD1718	<i>lacZ::Ω(recA_{E. coli}::lux_{p. l.})</i>

5

EXAMPLE 10IDENTIFICATION OF MUTAGENIC COMPOUNDSvs. STANDARD AMES REVERTANT ASSAY

Example 10 compares the sensitivity and accuracy of a screen for
10 mutagenic compounds using a *recA-LUX* containing detector cell as opposed to a standard revertant based Ames test.

The example evaluated the mutagenic potential of the submitted test
substances in *Salmonella typhimurium* strains TA100, TA1535, TA97a, and TA98
and in *Escherichia coli* strain WP2 *uvrA* (pKM101). The *Salmonella* strains are
15 unable to synthesize histidine, an essential amino acid, because of mutations in the
genes coding for histidine biosynthetic enzymes. Additional mutations in the
defective genes can result in individual *Salmonella* bacteria regaining the ability
to synthesize histidine [(Maron, D. M. and B. N. Ames, *Mutation Research* 113,

173-215, (1983)]. *E. coli* WP2 uvrA (pKM101) is unable to synthesize tryptophan due to an ochre mutation in a gene required for tryptophan biosynthesis. *E. coli* reversion mutants may arise either from further changes at the ochre site or from suppressor mutations at a locus in tRNA genes. [Brusick et al., *Mutation Research* 76, 169-190, (1980)]. A trace of histidine or tryptophan in the top agar permits several generations of auxotrophic cell division to fix pro-mutagenic lesions. This results in the formation of a microscopic "lawn" of bacteria. However, only those bacteria that revert to a prototrophic state can continue to divide and form macroscopic colonies. These revertants can be scored by their ability to grow on agar plates deficient in histidine (*S. typhimurium* strains) or tryptophan (*E. coli*). By comparing the number of chemically induced revertants to the number of spontaneous revertants, the mutagenicity of the test substance can be assessed.

Test Substance and Negative Control

15 Dimethyl sulfoxide (DMSO) was the solvent used for all compounds tested and thus was chosen as the test substance solvent and as the negative control. Dilutions of the test substance were performed at room temperature in DMSO. The negative control was assumed to be stable during this study, and no evidence of instability was observed. Any impurities were not expected to have interfered with the study.

Positive Indicators

Positive indicators included the following: 2-aminoanthracene (2AA), 2-nitrofluorene (2NF), sodium azide (NAAZ), ICR 191 Acridine (ICR 191), and methyl methanesulfonate (MMS). Deionized water was the solvent for NAAZ, ICR 191, and MMS. The solvent for other positive indicators was DMSO. The positive indicators were assumed to be stable in this study and no evidence of instability was observed. Any impurities were not expected to have interfered with the study.

Salmonella Tester Strain Characterization

30 *S. typhimurium* tester strains were obtained from Dr. Bruce Ames, Berkeley, CA. The phenotypic characteristics and mutational sensitivities of the *S. typhimurium* strains are summarized in Table 14 as follows:

TABLE 14

Strain	His Gene Locus affected	DNA Repair	LPS	R-factor plasmid	Mutational sensitivity
TA100	<i>hisG46</i>	Δ uvrB	<i>rfa</i>	Present	Base pair substitution
TA1535	<i>hisG46</i>	Δ uvrB	<i>rfa</i>	Absent	Base pair substitution
TA97a	<i>hisD6610</i>	Δ uvrB	<i>rfa</i>	Present	Frameshift
TA98	<i>hisD3052</i>	Δ uvrB	<i>rfa</i>	Present	Frameshift

The deletion in *uvrB* (a gene which codes for DNA excision repair) increases the bacterial sensitivity to mutagens. [Ames, B. N., F. D. Lee, and W. E. Durston, *Proc. Natl. Acad. Sci. USA* 70, 782-786, (1973)]. The *uvrB* trait is confirmed by demonstrating an increased bacterial sensitivity to ultraviolet light. Because this deletion also extends through a gene needed for biotin biosynthesis, the bacteria require exogenous biotin for growth. The *rfa* mutation causes a partial loss in the integrity of the lipopolysaccharide (LPS) cell wall so that permeability to large molecules is increased (Ames et al., 1973). The presence of the R-factor plasmid (plasmid pKM101 which carries ampicillin resistance as a marker gene) results in an enhancement of an error-prone DNA repair system which is endogenous to these bacteria. [McCann et al., *Proc. Natl. Acad. Sci. USA* 72, 979-983, (1975)]

Although the testing guidelines cited require *S. typhimurium* strain TA1537, the more recently developed TA97a strain was used. TA97 was the recommended replacement for TA1537 and has been demonstrated to be more sensitive to frameshift mutagens.(1,5) However, the reconstructed strain, TA97a, is now routinely used in place of TA97 due to its improved growth properties (personal communication with Bruce Ames and associates).

E. coli WP2 *uvrA* (pKM101) was obtained from the National Collection of Industrial Bacteria, Torrey Research Station, Scotland. Because tryptophan biosynthesis is blocked by an ochre nonsense mutation, revertants arise as a result of base pair substitution. A second class of mutants may arise as a result of nonsense suppressor mutations in genes coding for tRNAs. Frameshift mutagens are not generally expected to be detected by this strain. (Brusick, et al. *supra*)

Salmonella Tester Strain Storage and Culture

All bacterial strains were stored frozen in 8% DMSO in Oxoid nutrient broth at approximately -70°C. Overnight cultures were prepared by inoculating 20 mL of Oxoid nutrient broth with 0.1 mL of thawed bacterial suspension and incubating at 37°C with shaking. Overnight cultures were then stored on ice until

used for mutagenesis assays. Bacterial strain phenotypes were confirmed on previous stored isolates and not concurrently with each trial. Results for the frozen permanent stock of strains has demonstrated the appropriate responses.

Dose Selection

- 5 The highest concentration evaluated was a solution or suspension of the test substance at concentrations of 100 µg/plate. Solubility information was confirmed empirically, as needed. Concentrations were calculated with the assumption that addition of the test substance to the solvent did not change the volume of the resulting solution.

10 Stability And Concentration Verification

- Solutions of the test substance were prepared immediately prior to treatment and were presumed to be stable under the conditions of the study. Treatment and control dosing solutions were not analyzed for concentration, uniformity, or stability. Top agar was not assayed for stability or concentration of
15 the test substance or control articles since this assessment was not considered necessary to achieve the objectives of the study.

Bacterial Mutagenicity Assays

- This study consisted of one trial without metabolic activation. Three replicates were plated for each tester strain, test concentration, and condition.
20 Positive indicators and negative controls were included in all assays. Treatments with activation were conducted by adding 0.1 mL of negative control or test substance solution, 0.5 mL of S9 mix, and 0.1 mL of an overnight culture containing at least 1 x10⁸ bacteria to 2 mL of top agar [0.6% agar (w/v) and 0.6% NaCl (w/v)] supplemented with 0.05 mM L-histidine and 0.05 mM D-biotin for S.
25 typhimurium strains or 0.05 mM L-tryptophan for the *E. coli* strain. These components were mixed and poured onto a plate containing 25 mL of Davis minimal agar with dextrose (minimal agar plates, purchased from MOLTOX. Revertant colonies were counted after the individually labeled plates were incubated at approximately 37°C for about 48 hours. When necessary, plates were
30 refrigerated prior to counting.

Statistical Analysis

For each tester strain, the average number of revertants and the standard deviation at each concentration were calculated.

Classification Guidelines

- 35 A test substance was classified as positive when: (1) the average number of revertants in any strain at any test substance concentration studied was at least two times greater than the average number of revertants in the negative control; and (2) there was a positive dose-response relationship in that same strain.

A test substance was classified as negative when either: (1) there were no test substance concentrations with an average number of revertants which was at least two times greater than the average number of revertants in the negative control; and (2) there was no positive dose-response relationship.

Test substances, AA, BB, CC, DD, EE, FF, and GG, were evaluated for mutagenicity in *Salmonella typhimurium* strains TA100, TA1535, TA97a, and TA98 and in *Escherichia coli* strain WP2 uvrA (pKM101) without an exogenous metabolic activation system (S9).

Concentrations of 6.25, 12.5, 25.0, 50.0 and 100.0 (g/plate) were evaluated in comparison to negative (solvent) controls. Under the conditions of these studies, evidence of mutagenic activity was detected in five of the seven test articles assessed. Results are summarized in Table 15 below:

TABLE 15

Lowest conc. in µg/ml:

compound	inducing luminescence ^(a)	preventing luminescence ^(a)	for mutation ^(b)	for growth inhibition ^(b)
AA	0.3	1.3	50	100
BB	-	5.0	-	----
CC	0.1	0.6	6	100
DD	0.6	1.3	-	6
EE	0.6	-	6	25
FF	1.3	2.5	100	
GG	0.3	1.3	6	13
(1)	(2)	(3)	(4)	(5)

(a) Assay volume of 100ul

(b) Plate volume of 28ml

AA, BB, CC, EE, FF, and GG displayed evidence of mutagenic activity. Due to what was judged as test-substance related toxicity, there were insufficient acceptable concentrations to assess the mutagenicity of DD.

Five of the six light inducing compounds (col. 2) are also mutagenic (col. 4) while the threshold for inducing light appears equal or lower (col. 2) than that causing mutation (col. 4). Moreover the lights off assay (col. 3) appears superior to the growth inhibition assay (col. 5).

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT:
 - (A) ADDRESSEE: E. I. DU PONT DE NEMOURS AND COMPANY
 - (B) STREET: 1007 MARKET STREET
 - (C) CITY: WILMINGTON
 - (D) STATE: DELAWARE
 - (E) COUNTRY: U.S.A.
 - (F) ZIP: 19898
 - (G) TELEPHONE: 302-892-8112
 - (H) TELEFAX: 302-773-0164
 - (I) TELEX: 6717325
- (ii) TITLE OF INVENTION: A METHOD FOR IDENTIFYING THE
SITE OF ACTION OF XENOBIOTIC
CHEMICALS
- (iii) NUMBER OF SEQUENCES: 10
- (iv) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: DISKETTE, 3.50 INCH
 - (B) COMPUTER: IBM PC COMPATIBLE
 - (C) OPERATING SYSTEM: MICROSOFT WINDOWS 95
 - (D) SOFTWARE: MICROSOFT WORD VERSION 7.0A
- (v) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (vi) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: 60/039,582
 - (B) FILING DATE: FEBRUARY 28, 1997
- (vii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: FLOYD, LINDA AXAMETHY
 - (B) REGISTRATION NO.: 33,692
 - (C) REFERENCE/DOCKET NUMBER: CR-9952

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ACTTTTAAGC TTAGAGAAGC CTGTCGGCAC

30

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 29 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

AGCTTTGGAT CCCGCTTCT GTTTGTTTT

29

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GCCACTATCG ACTACGCG

18

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CTGTGGCGCC GGTGATGC

18

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(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GTAAAACGAC GGCCAGT

17

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

AGCGGATAAC AATTTACAC AGGA

24

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GTTAGCGGAT CCCTAATTT AGCCTCAGCC

30

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

AGCAGTGAAT TCGTGTATCG TGCATCACTT C

31

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(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GTTAGCGGAT CCTAATAGGG GCATTCTCCG

30

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

AGCAGTGAAT TCCAGCCGAT ATAGTAACGA CAG

33

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CLAIMS:

1. A method for the identification of the site of action of a stressor comprising:
 - (i) contacting a stressor with a detector bacteria, the detector
 - 5 bacteria comprising a genotoxic-sensitive promoter operably linked to a luminescent reporter gene complex to form a gene fusion that confers a bioluminescent positive phenotype upon the detector bacteria wherein exposure of the genotoxic-sensitive promoter to genotoxic compounds drives heightened expression of the luminescent reporter gene complex producing an increased
 - 10 bioluminescent signal;
 - (ii) selecting for genotoxic or non-genotoxic stressors capable of inhibiting the growth of the detector bacteria of step (i) by monitoring the growth and light output of the detector bacteria;
 - (iii) submitting the growth-inhibiting, genotoxic or non-genotoxic
 - 15 stressor selected in to a site of action screen;
 - (iv) identifying the site of action in the detector bacteria; and
 - (v) confirming the site of action in a target organism.
 2. The method of Claim 1 wherein the stressor of step (ii) is genotoxic and the stressor of step (iii) is non-genotoxic.
 3. The method of Claim 1 wherein the luminescent reporter gene
 - 20 complex is selected from the group consisting of thermostable *lux*, *luxCDABE*, thermolabile *lux*, *renella lux*, and *luc*.
 4. The method of Claim 1 wherein the detector bacteria is an enteric bacteria and comprises a first mutation conferring the ability to accumulate crop
 - 25 protection chemicals and a second mutation preventing a global cellular response to the active stressor.
 5. The method of Claim 4 wherein the second mutation prevents the global cellular response to amino acid starvation.
 6. The method of Claim 1 wherein the stressor is selected from the
 - 30 group consisting of crop protection chemicals, antibacterial chemicals, antiviral chemicals, anticancer chemicals, and anticorrosive chemicals.
 7. The method of Claim 6 wherein the stressor is selected from the group of crop protection chemicals consisting of herbicides, pesticides, and fungicides.
 8. The method of Claim 7 wherein the stressor is selected from the
 - 35 group consisting of sulfonylurea herbicides, profungicides, fungicides, oxidants, and hormonal herbicides.
 9. The method of Claim 8 wherein the stressor is selected from the group consisting of Sulfometuron methyl, glyphosate, oxime carbamates, methyl

viologen, 2,4-dichlorophenoxyacetic acid, 2-thienylalanine, acivicin, and mitomycin C.

10. The method of Claim 1 wherein the genotoxic-sensitive promoter is selected from the group of genotoxic-sensitive promoters controlled by the SOS
5 regulatory circuit.

11. The method of Claim 10 wherein the genotoxic-sensitive promoter is selected from the group of genotoxic-sensitive promoters consisting of *recA*, *lexA*, *umuDC*, *uvrA*, *uvrB*, *uvrC*, *sulA*, *recN*, *uvrD*, *ruv*, *dinA*, *dinB*, *dinD*, and *dinF*.

12. The method of Claim 11 wherein the genotoxic-sensitive promoter is
10 *recA*.

13. The method of Claim 1 wherein the site of action screen is a genetic titration screen or a nutritional reversal screen.

14. A detector cell strain comprising a *recA-LuxCDABE* gene fusion selected from the group consisting of DPD1707, DPD1708, DPD1715, DPD1716,
15 DPD1718, DPD1719 DPD1728, DPD1729, and DPD1730.

15. A non-bioluminescent parent strain selected from the group consisting of DPD1678, DPD1675, DPD1690 DPD1012, DPD1013, DPD1692, DPD1680, DPD1693, DPD1682 DPD1010 and DPD1011.

16. A method for the identification of thienylalanine-like compounds
20 comprising:

(i) contacting a compound suspected of having thienylalanine-like activity with a first detector bacteria, and with a second detector bacteria, the first detector bacteria comprising:

a) an expressible *luxCDABE* gene fusion; and
25 b) the normal cellular complement of a gene selected from the group consisting of *pheA* and *aroH* wherein the gene is not overexpressed; and the second detector cell comprising:

a) an expressible *luxCDABE* gene fusion; and
b) multiple copies of an expressible gene selected from the
30 group consisting of *pheA* and *aroH* wherein the expressible gene is overexpressed; the first and second detector cells being isogenic and wherein the expressible *luxCDABE* gene fusion generates baseline luminescence;

(ii) monitoring the light output of the first and second detector cells contacted with a compound suspected of having thienylalanine-like whereby

35 a) light output equal to baseline luminescence in both the first and the second detector cells indicates a compound with no biological activity;

b) a decrease in light output as compared with baseline luminescence in both the first and the second detector cells indicates a compound with growth inhibitory activity and;

5 c) a decrease in light output in the first detector cell as compared with baseline luminescence and an increase or maintenance of light output in the second detector cell indicates a compound having thienylalanine-like activity.

17. A method for the identification of Glyphosate-like compounds comprising:

10 (i) contacting a compound suspected of having glyphosate-like activity with a first detector bacteria and with a second detector bacteria, the first detector bacteria comprising:

- a) an expressible *luxCDABE* gene fusion; and
- b) the normal cellular complement of an *aroA* gene wherein

15 the *aroA* gene is not overexpressed

the second detector cell comprising:

- a) an expressible *luxCDABE* gene fusion; and
- b) multiple copies of an expressible *aroA* gene selected

wherein the *aroA* gene is overexpressed;

20 the first and the second detector cells being isogenic and wherein the expressible *luxCDABE* gene fusion generates baseline luminescence;

(ii) monitoring the light output of the first and the second detector cells contacted with a compound suspected of having glyphosate-like activity whereby

25 a) light output equal to baseline luminescence in both the first and the second detector cells indicates a compound with no biological activity;

b) a decrease in light output as compared with baseline luminescence in both the first and the second detector cells indicates a compound with growth inhibitory activity; and

30 c) a decrease in light output in the first detector cell as compared with baseline luminescence and an increase or maintenance of light output in the second detector cell indicates a compound having glyphosate-like activity.

35 18. A method for the identification of ALS-inhibiting compounds comprising:

(i) contacting a compound suspected of having ALS-inhibiting activity with a first detector bacteria and with a second detector bacteria, the first detector bacteria comprising:

a) an expressible *luxCDABE* gene fusion; and
 b) the normal cellular complement of a gene selected from the group consisting of *ilvBN* and *ilvIH* wherein the gene is not overexpressed;
 the second detector cell comprising:

- 5 a) an expressible *luxCDABE* gene fusion; and
 b) multiple copies of an expressible gene selected from the group consisting of *ilvBN* and *ilvIH* wherein the expressible gene is overexpressed;

the first and the second detector cells being isogenic and wherein the
 10 expressible *luxCDABE* gene fusion generates baseline luminescence;

(ii) monitoring the light output of the first and the second detector cells contacted with a compound suspected of having ALS-inhibiting activity whereby

a) light output equal to baseline luminescence in both the
 15 first and the second detector cells indicates a compound with no biological activity;

b) a decrease in light output as compared with baseline luminescence in both the first and the second detector cells indicates a compound with growth inhibitory activity; and

20 c) a decrease in light output in the first detector cell as compared with baseline luminescence and an increase or maintenance of light output in the second detector cell indicates a compound having ALS-inhibiting activity.

19. A method for identifying a genotoxic substance, comprising the steps
 25 of:

(i) culturing a detector cell comprising a promoter regulated by a SOS bacterial regulatory circuit and a *luxCDABE* gene complex wherein the *luxCDABE* gene complex is positioned in the bacterial chromosome downstream of the SOS promoter such that when the SOS promoter is expressed, then the
 30 *luxCDABE* gene complex is also expressed;

(ii) contacting the culture of step (i) with a substance to be tested; and

(iii) determining whether the substance is genotoxic by measuring the amount of luminescence in the culture.

35 20. The method of Claim 19 wherein the promoter regulated by a SOS bacterial regulatory circuit is selected from the group consisting of *recA*, *lexA*, *umuDC*, *uvrA*, *uvrB*, *uvrC*, *sulA*, *recN*, *uvrD*, *ruv*, *dinA*, *dinB*, *dinD*, and *dinF*.

21. A method of identifying a structural gene encoding a stressor target comprising:

- (i) contacting a stressor with a detector bacteria, the detector bacteria comprising a genotoxic-sensitive promoter operably linked to a luminescent reporter gene complex to form a gene fusion that confers a bioluminescent positive phenotype upon the detector bacteria wherein exposure of the genotoxic-sensitive promoter to genotoxic compounds drives heightened expression of the luminescent reporter gene complex producing an increased bioluminescent signal;
- (ii) selecting for genotoxic or non-genotoxic stressors capable of inhibiting the growth said detector bacteria of step (i) by monitoring the growth and light output of the detector bacteria;
- (iii) submitting the growth-inhibiting, genotoxic or non-genotoxic stressor to a site of action screen wherein the stressor target is identified and;
- (iv) isolating the structural gene encoding the stressor target.

22. A method for the identification of the site of action of a stressor comprising:

- (i) contacting a stressor with a detector bacteria, the detector bacteria comprising an expressible *luxCDABE* gene fusion wherein the expressible *luxCDABE* gene fusion generates baseline luminescence in the detector bacteria;
- (ii) selecting for stressors capable of inhibiting the growth of the detector bacteria of step (i) by monitoring the growth and light output of the detector bacteria;
- (iii) submitting the growth-inhibiting stressor selected in step (ii) to a site of action screen;
- (iv) identifying the site of action in the detector bacteria; and
- (v) confirming the site of action in a target organism.

23. The method of Claim 22 wherein the expressible *luxCDABE* gene fusion further comprises a stressor sensitive promoter selected from the group consisting of *grpE*, *dnaK*, *lon*, *rpoD*, *groESL*, *lysU*, *htpE*, *htpG*, *htpI*, *htpK*, *clpP*, *clpB*, *htpN*, *htpO*, *htpX*, *katG*, *ahp*, *micF*, *sodA*, *nfo*, *zwf*, *soi*, *fabA*, *his*, *ilvBN*, *ilvGMED*, *thrABC*, *lac*, *mal*, and *gal*, *phoA*, *phoBR*, *phoE*, *phoS*, *aphA*, *himA*, *pepN*, *ugpAB*, *psiD*, *psiE*, *psiF*, *psiK*, *psiG*, *psiI*, *psiJ*, *psiN*, *psiR*, *psiH*, *phiL*, *phiO*, *ara*, *tna*, *dsd*, *glnA*, and *hut*.

24. A method to identify a structural gene encoding a stressor target comprising:

- (i) contacting a stressor with a detector bacteria, the detector bacteria comprising a genotoxic-sensitive promoter operably linked to a

luminescent reporter gene complex to form a gene fusion that confers a bioluminescent positive phenotype upon the detector bacteria;

(ii) selecting for at least one stressor that inhibits the growth of the detector bacteria in step (i) by monitoring the growth and light output of the detector bacteria;

(iii) submitting the growth-inhibiting stressor selected in step (ii) to a nutritional reversal site of action screen comprising the steps of:

- (a) formulating a multiplicity of nutritional pools comprising minimal growth medium supplemented with a defined nutrient composition, each nutritional pool comprising a different nutrient composition from all other pools;
- (b) growing a detector bacteria of step (i) in each of the pools of (iii)(a) to create a nutrient culture; and
- (c) monitoring light output from the nutrient culture of (iii)(b) wherein an increase in light production indicates nutrient reversal; and

(iv) identifying the structural gene encoding a stressor target reversed by the nutrient of step (iii)(c).

25. A method to identify a structural gene encoding a stressor target comprising:

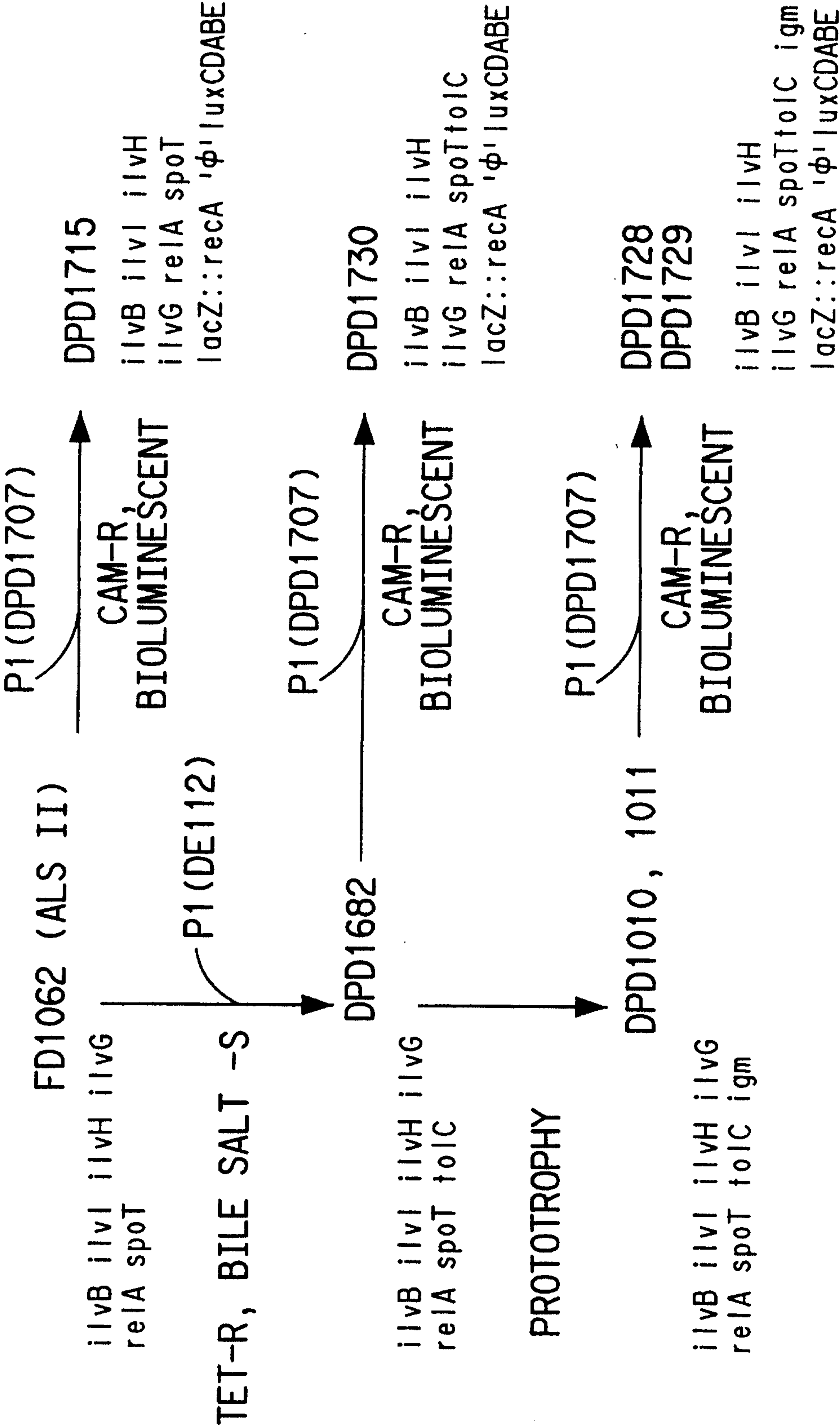
- (i) contacting a stressor with a detector bacteria, the detector bacteria comprising a genotoxic-sensitive promoter operably linked to a luminescent reporter gene complex to form a gene fusion that confers a bioluminescent-positive phenotype upon the detector bacteria;
- (ii) selecting for at least one stressor that inhibits the growth of the detector bacteria of step (i) by monitoring the growth and light output of the detector bacteria; and

(iii) submitting the growth-inhibiting stressor to a genetic titration site-of-action screen comprising the steps of:

- (a) constructing a genomic library in high-copy-number autonomously replicating plasmids from the genome of the detector organism of step (i);
- (b) transforming a suitable host cell with the genomic library of step (iii)(a);
- (c) contacting the transformed host of step (iii)(b) with a stressor to create a test culture; and
- (d) monitoring the test culture of step (iii)(c) for alterations in light production wherein an increase in luminescence

indicates the presence of a structural gene encoding a stressor target.

FIG. 1A



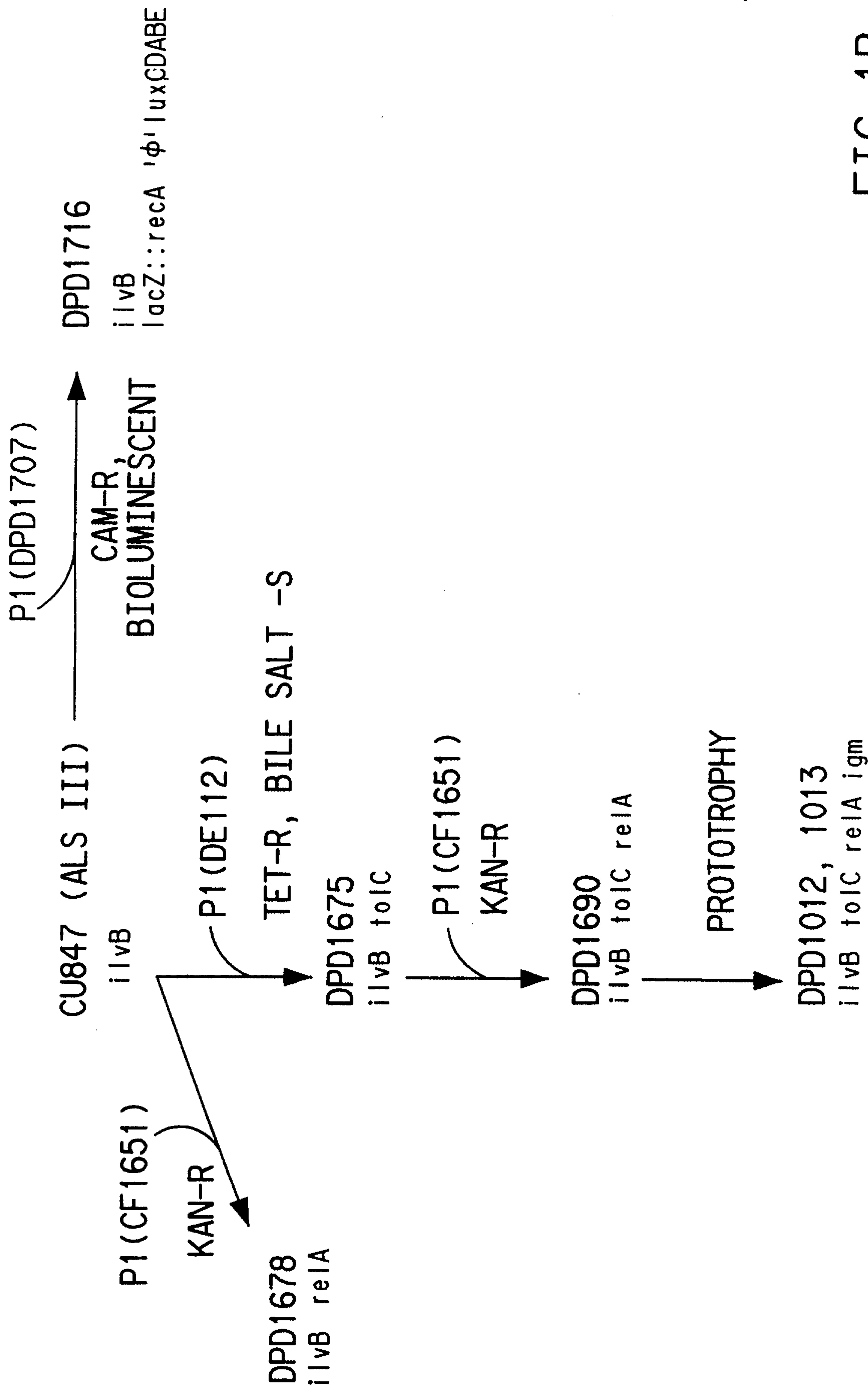


FIG. 1B

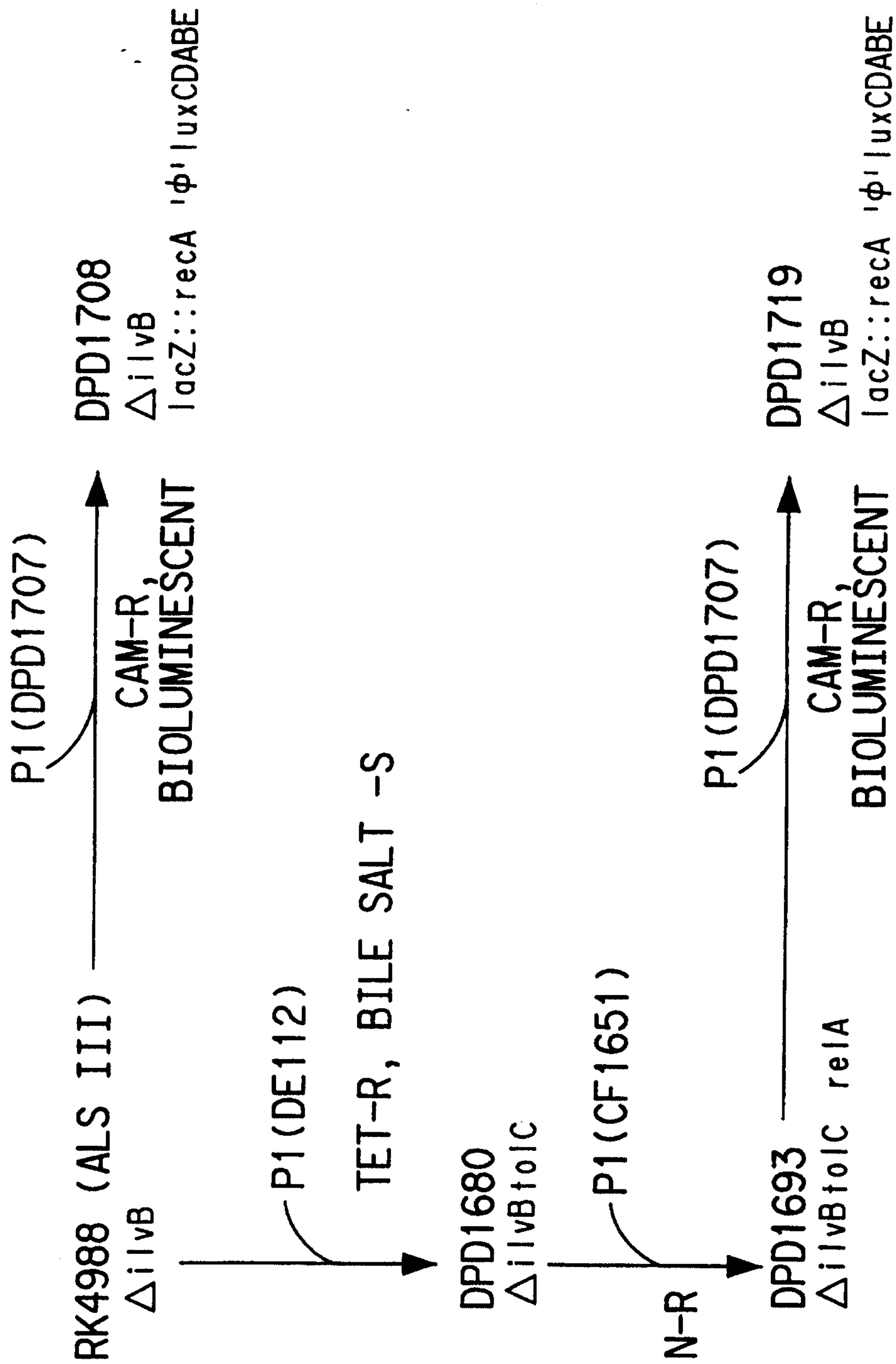
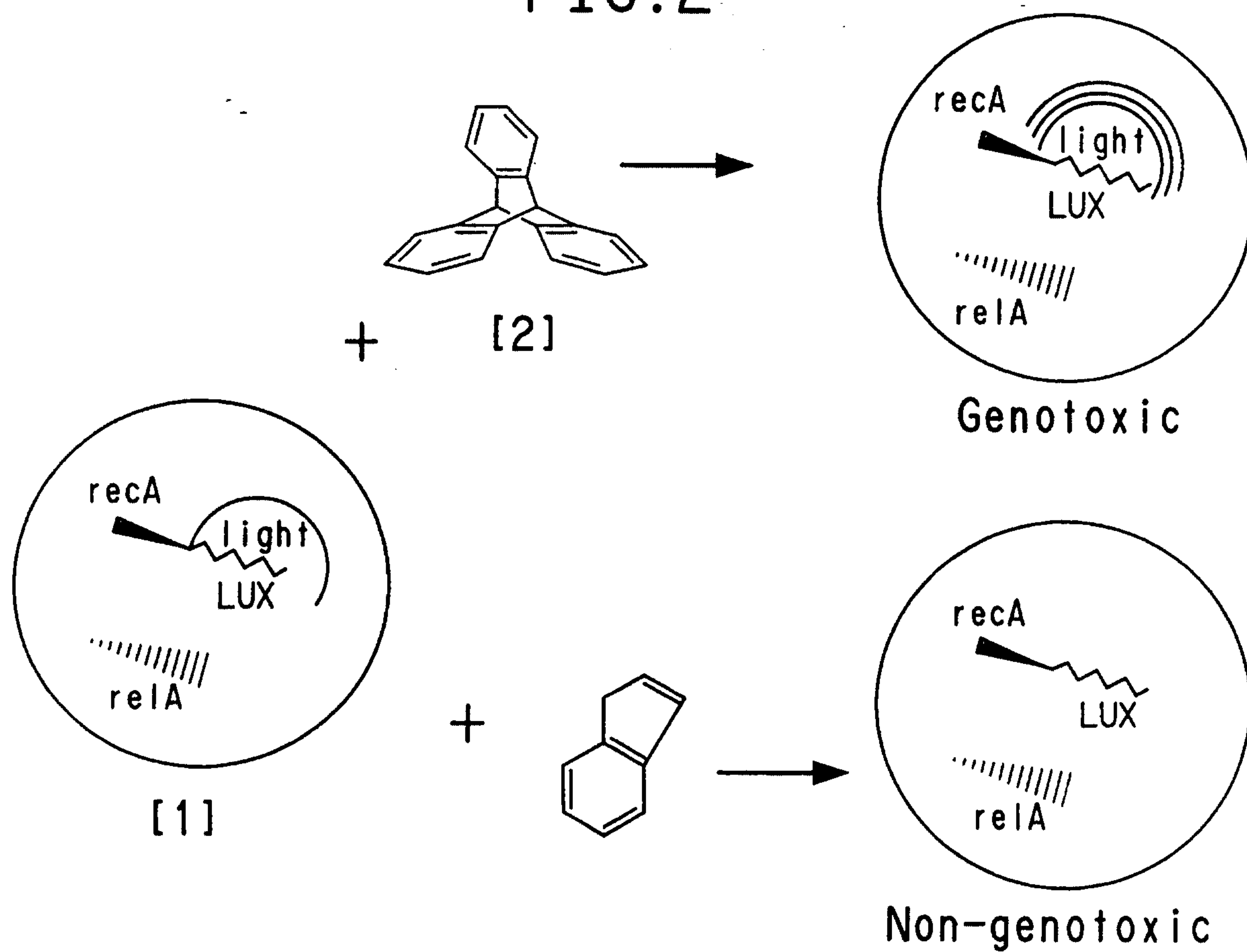


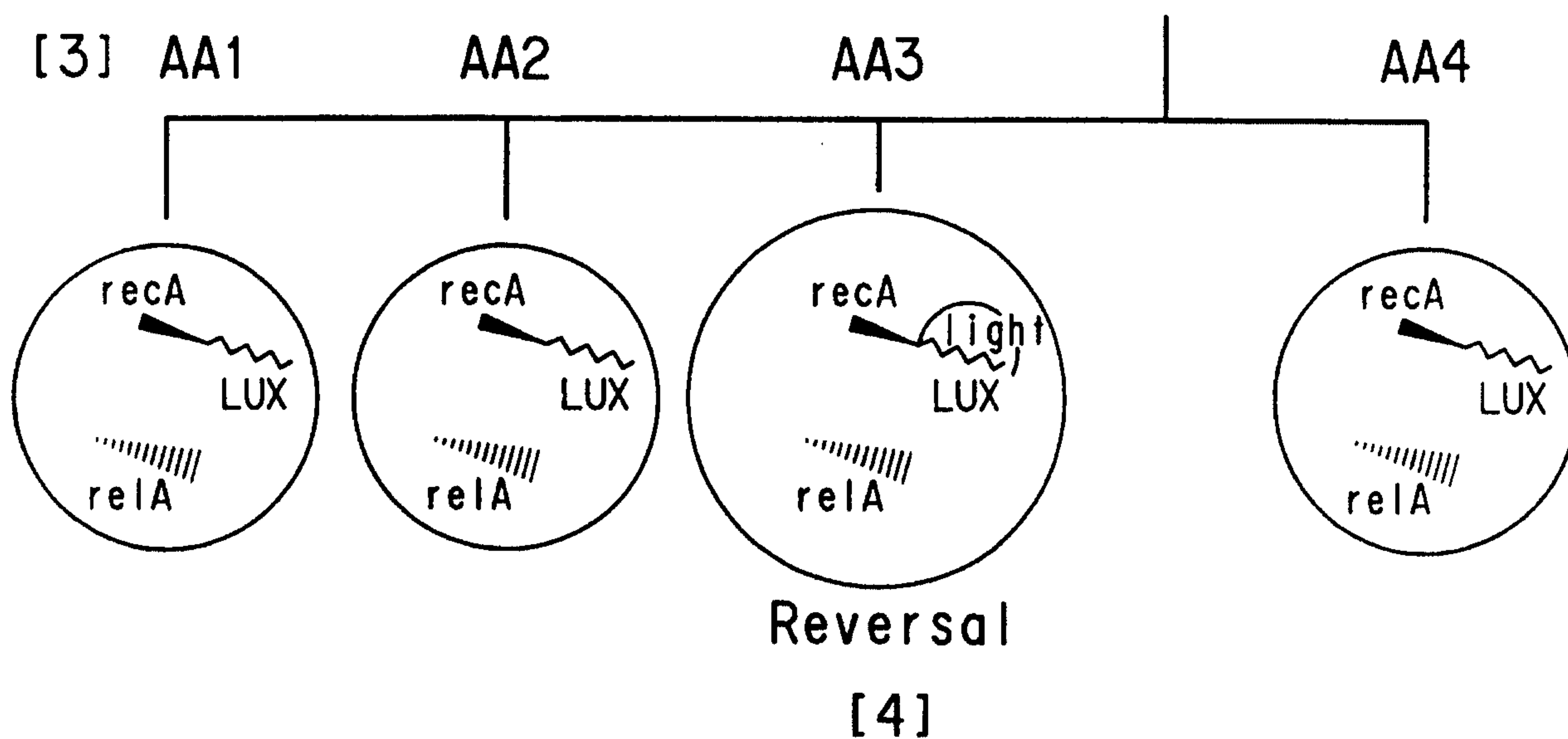
FIG. 1B (CONTINUED)

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FIG. 2

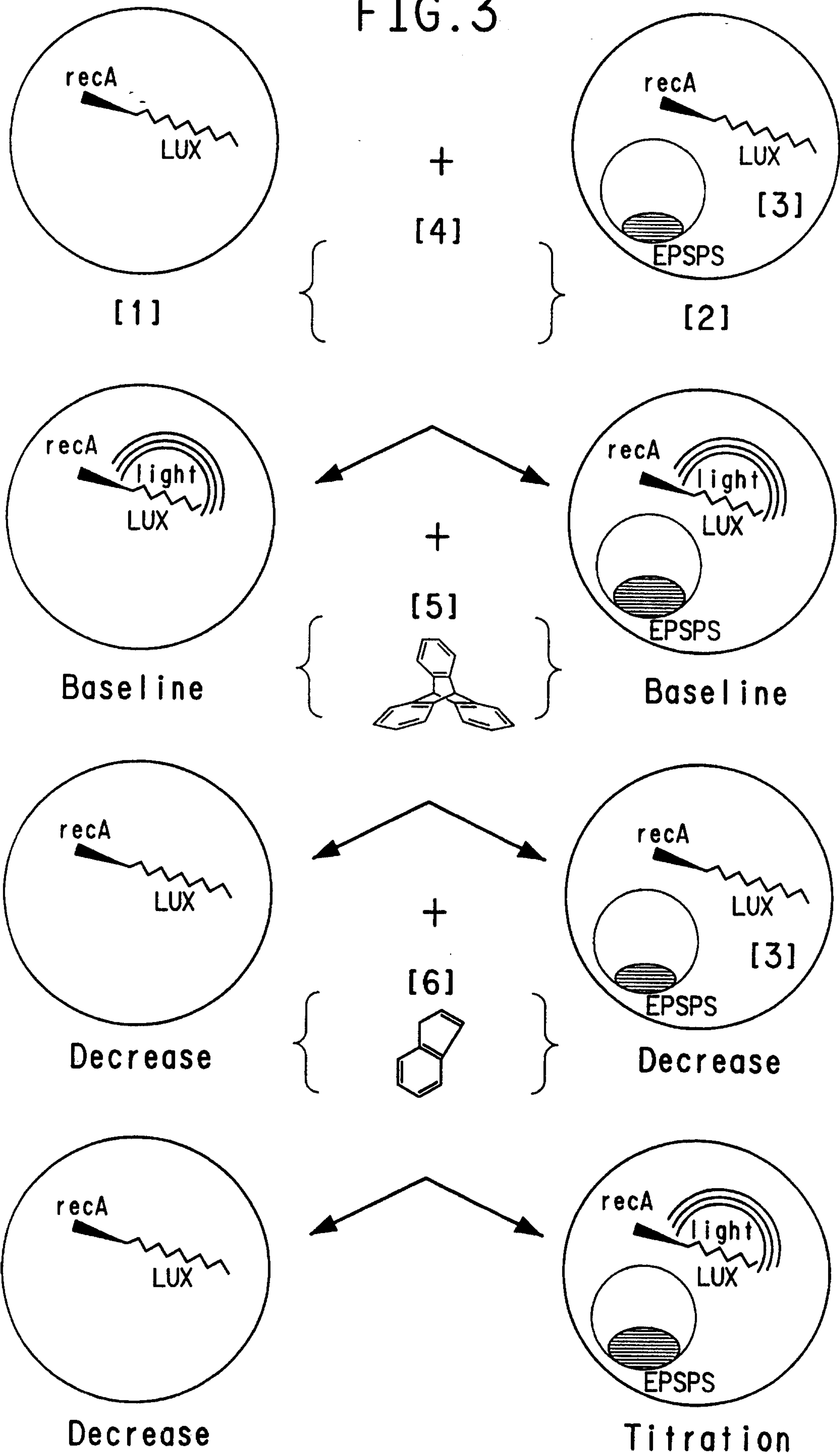


Minimal Medium Supplemented Pools



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FIG. 3



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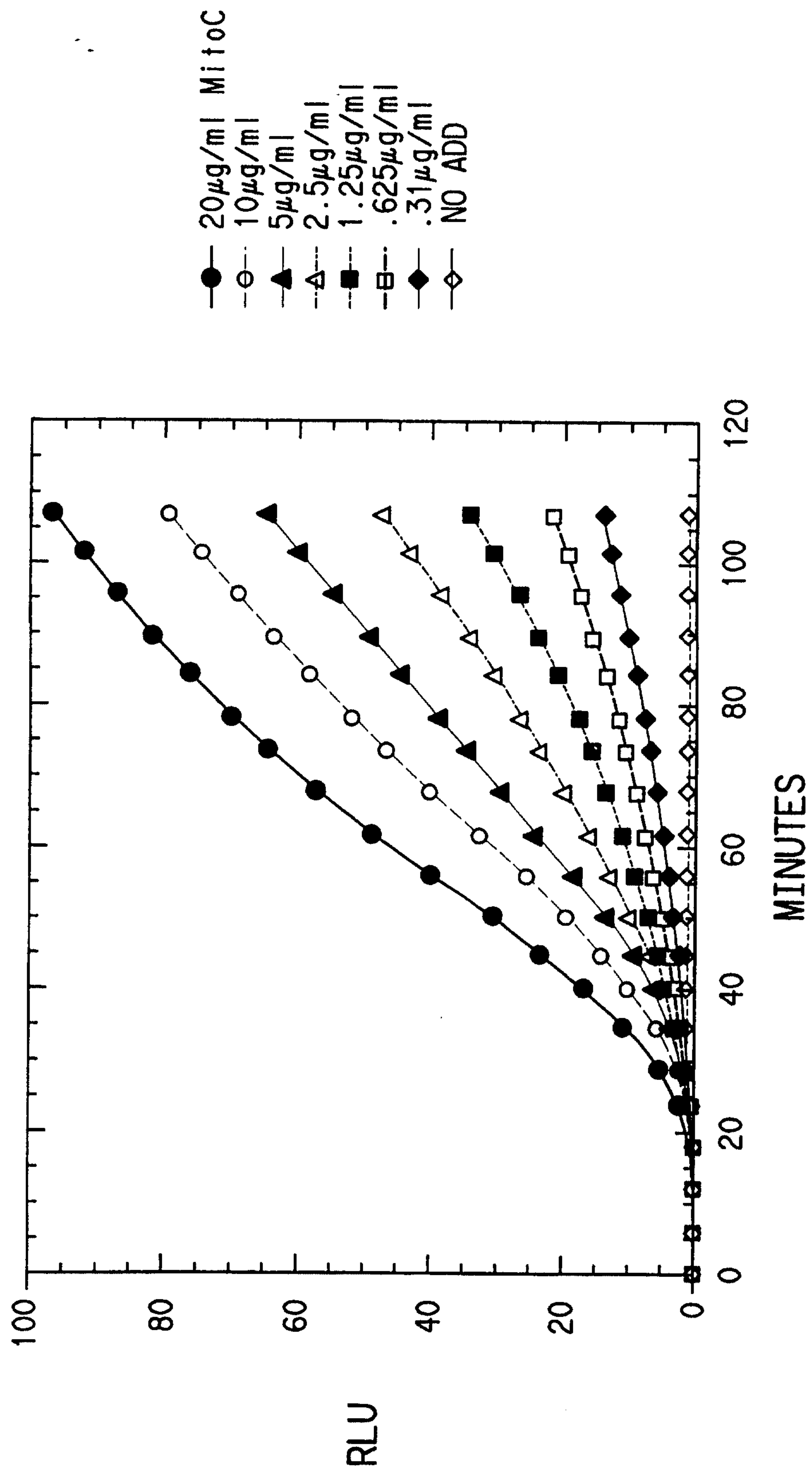


FIG. 4A

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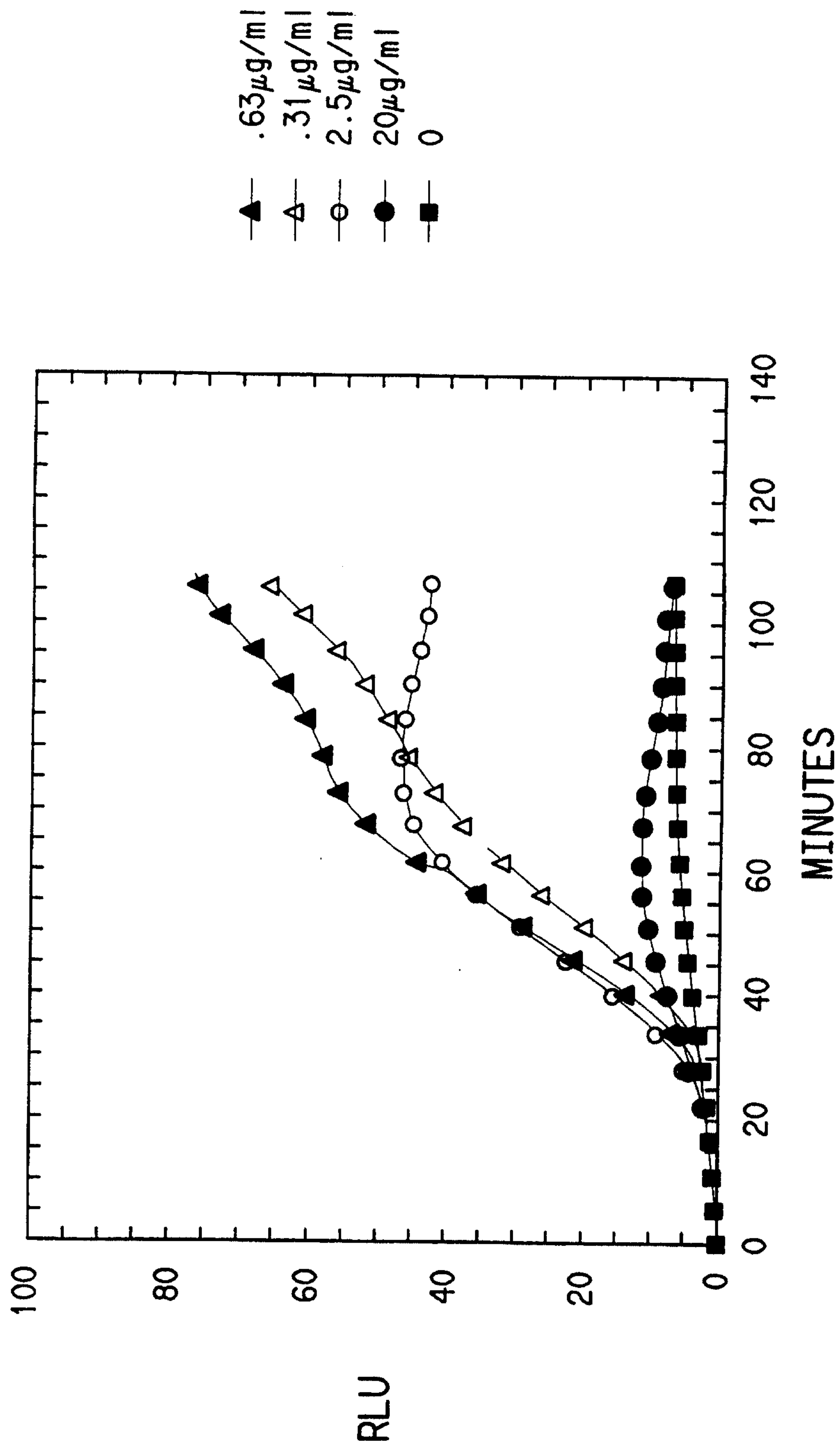


FIG. 4B

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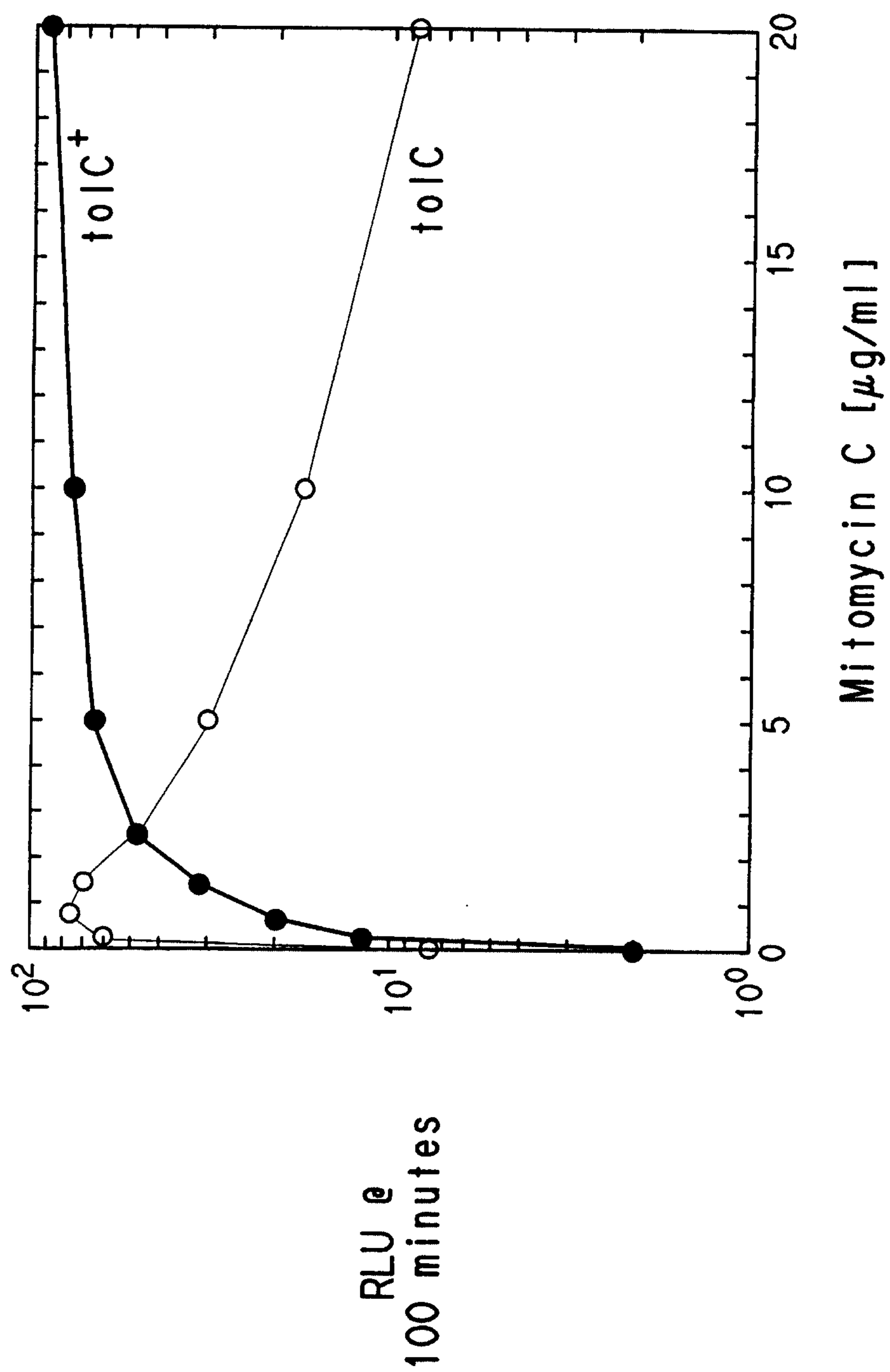


FIG.5

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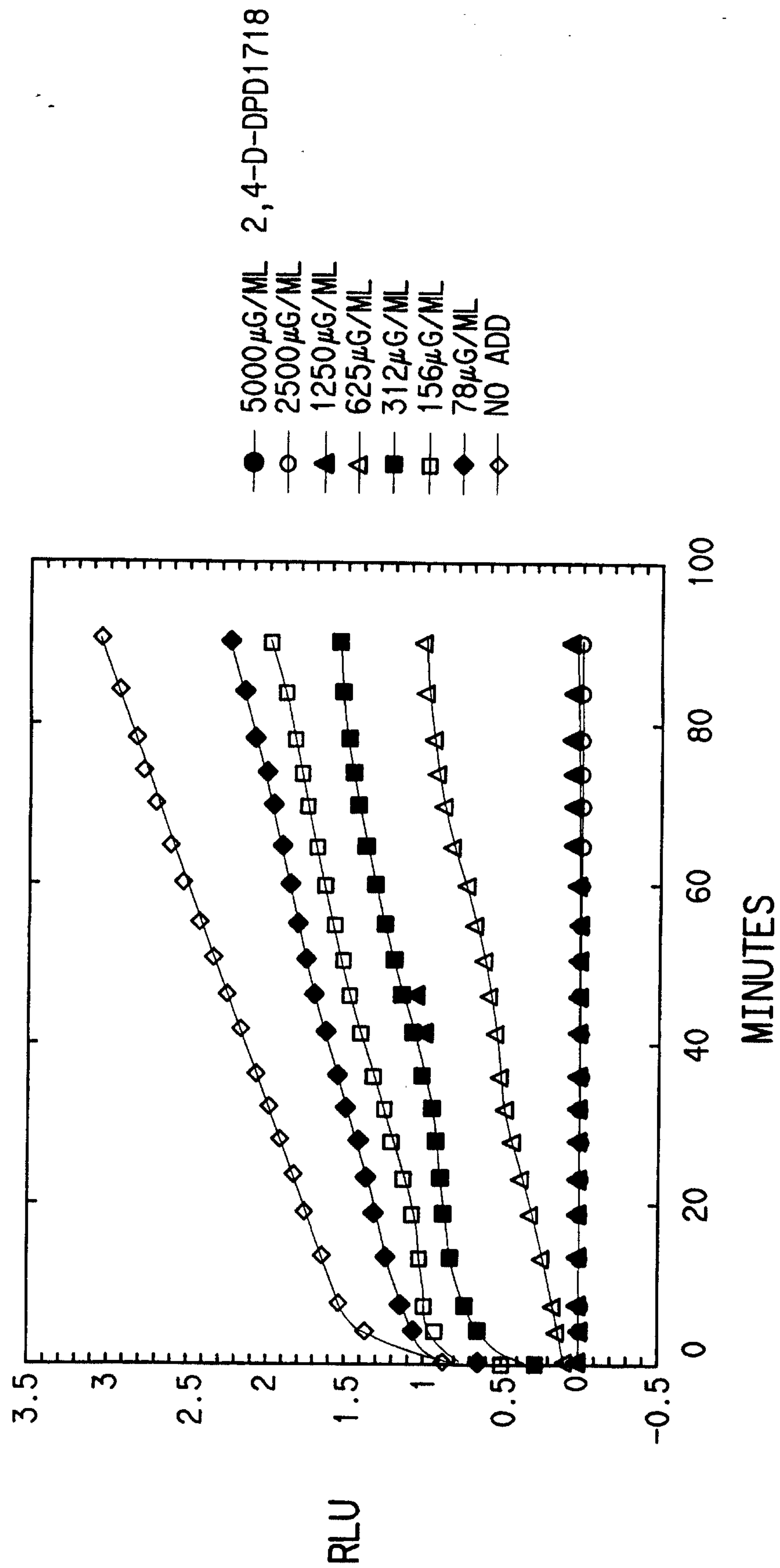


FIG. 6

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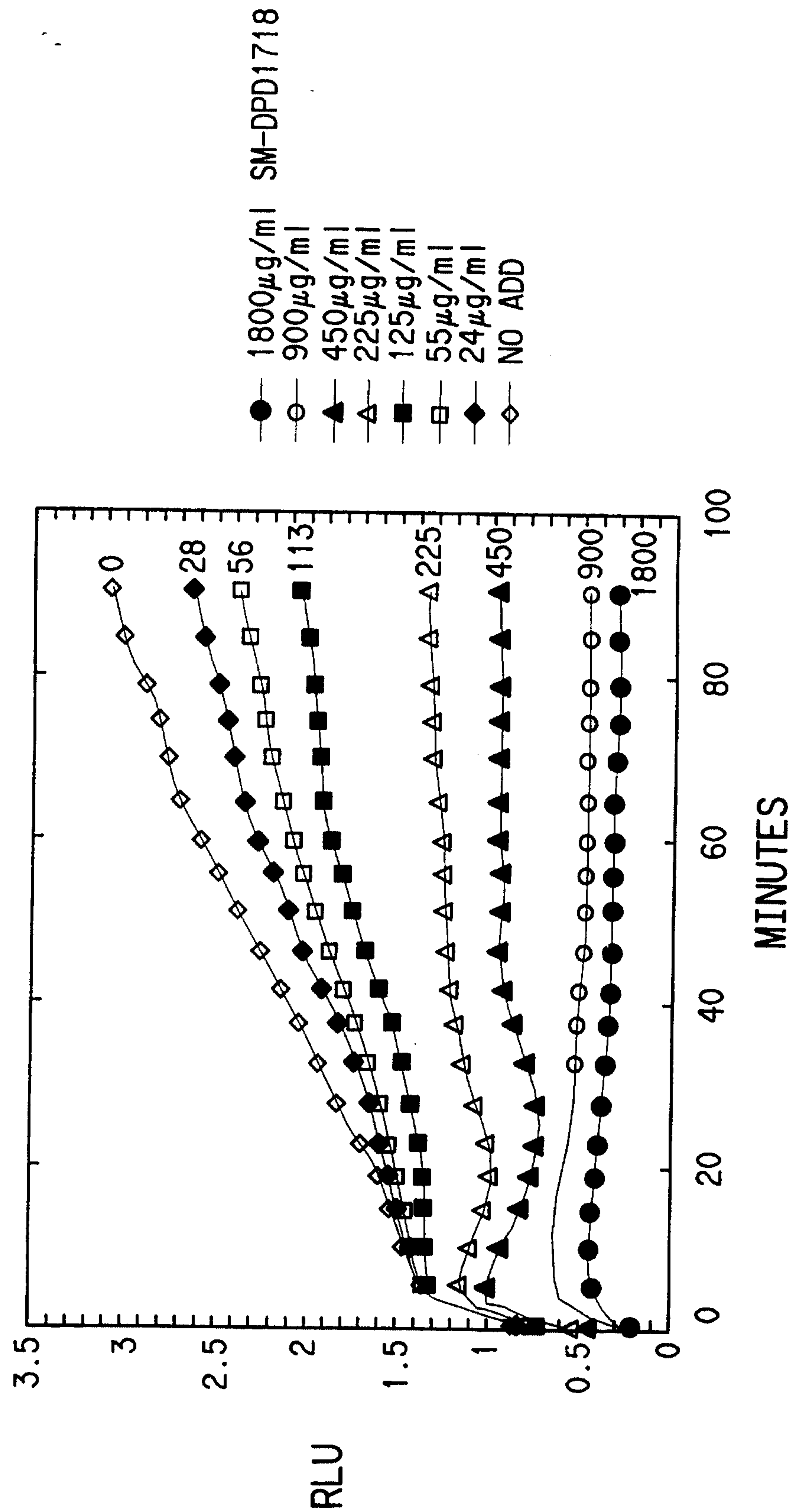


FIG. 7

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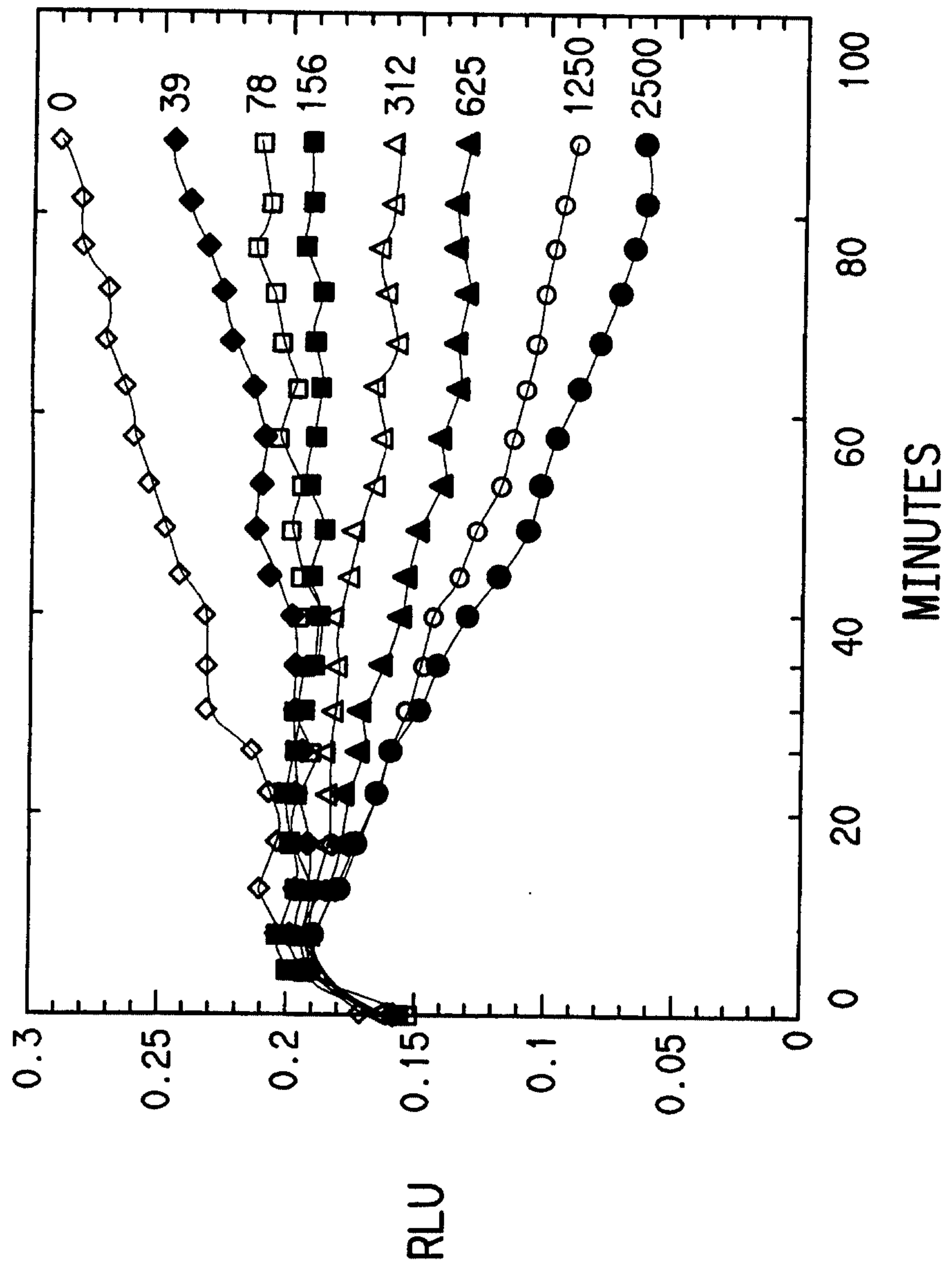


FIG. 8A

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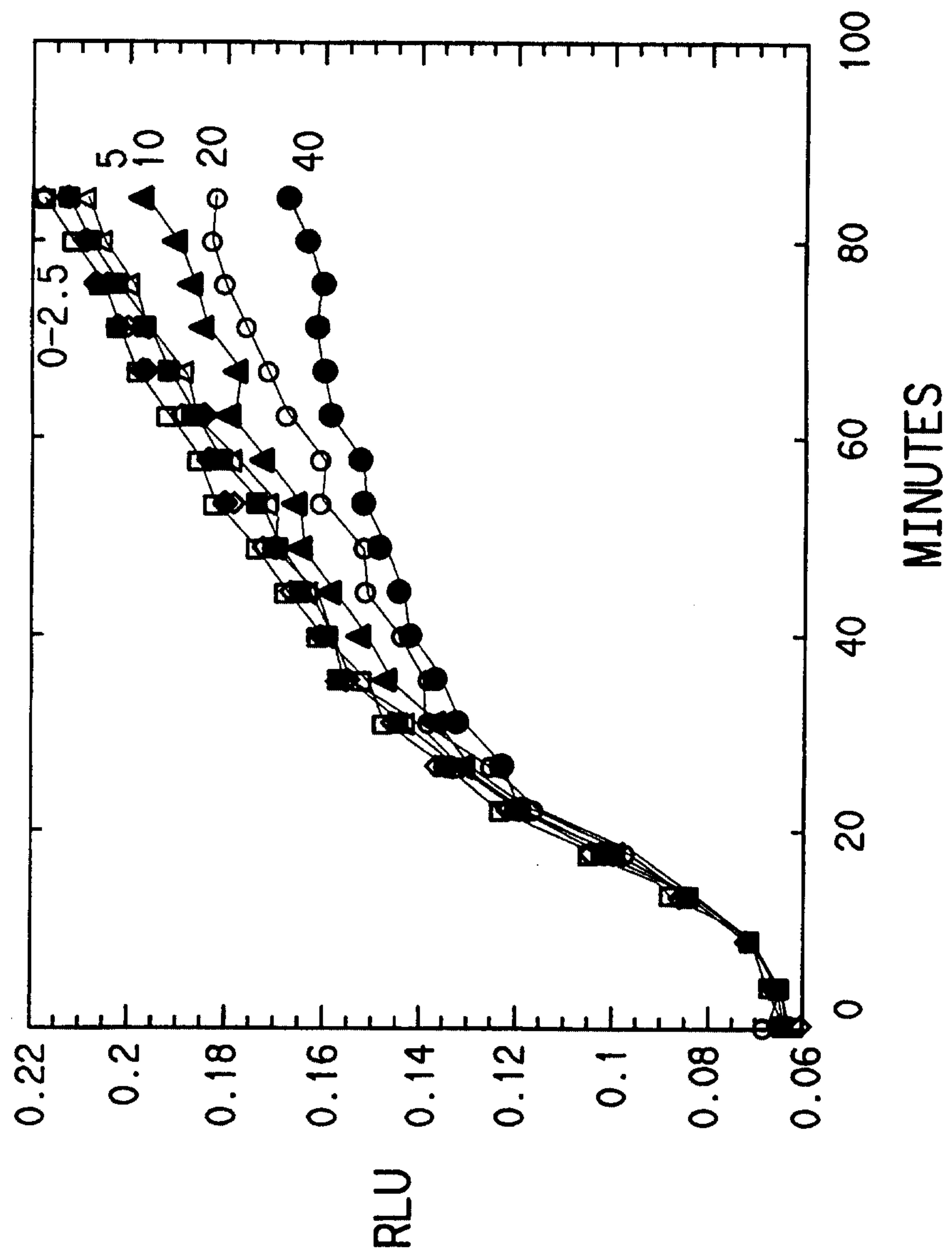


FIG. 8B

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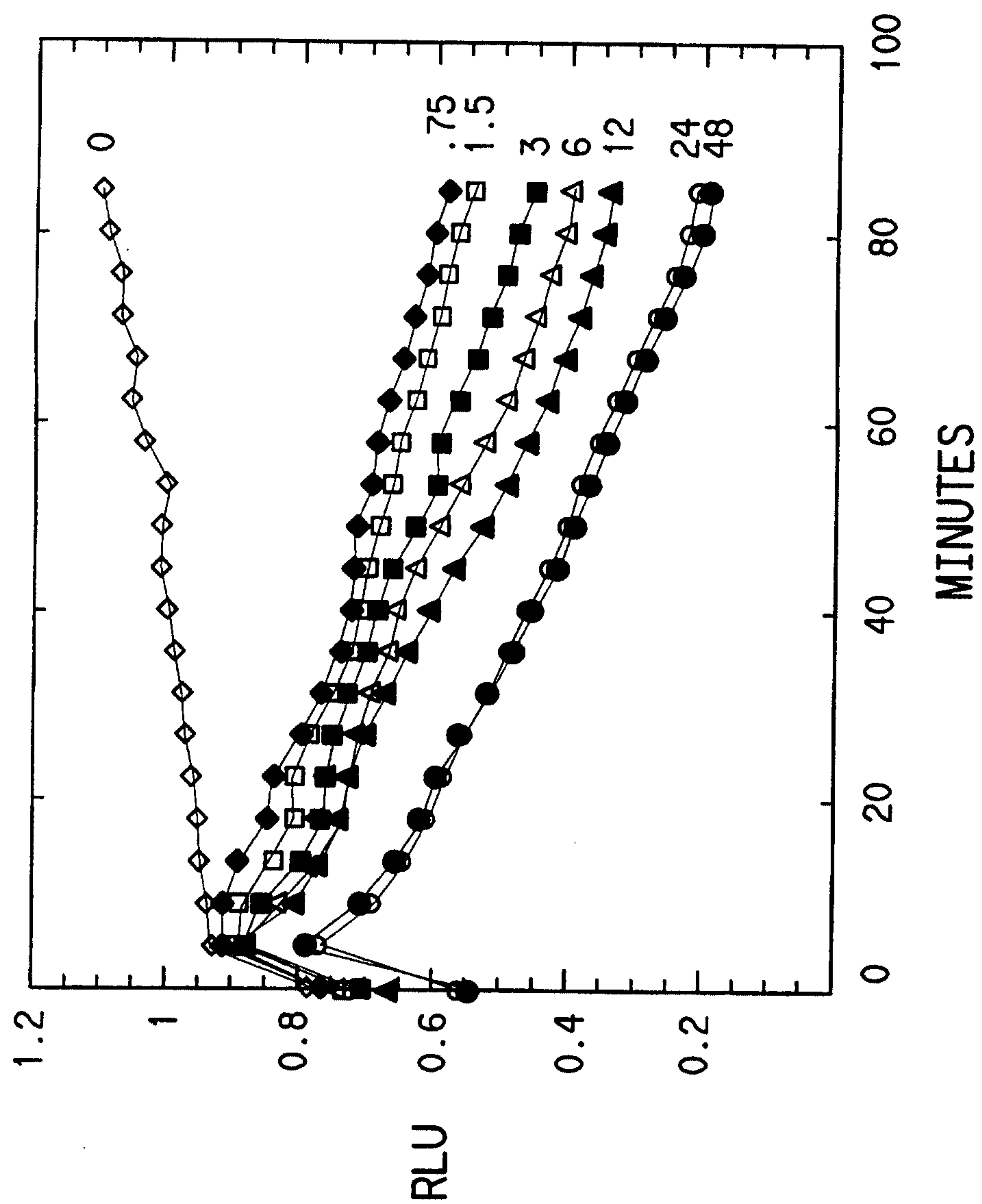


FIG. 9

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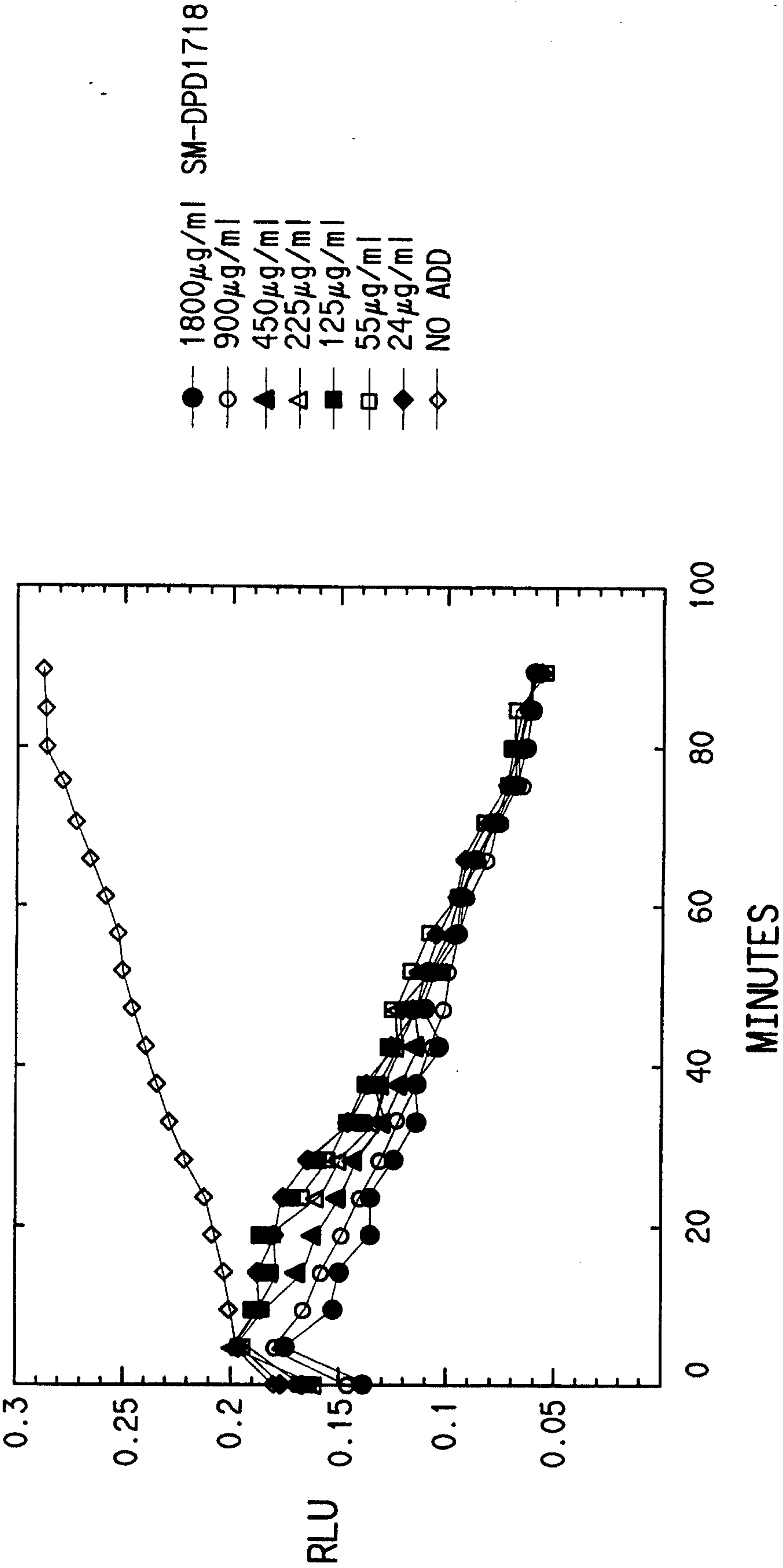


FIG. 10A

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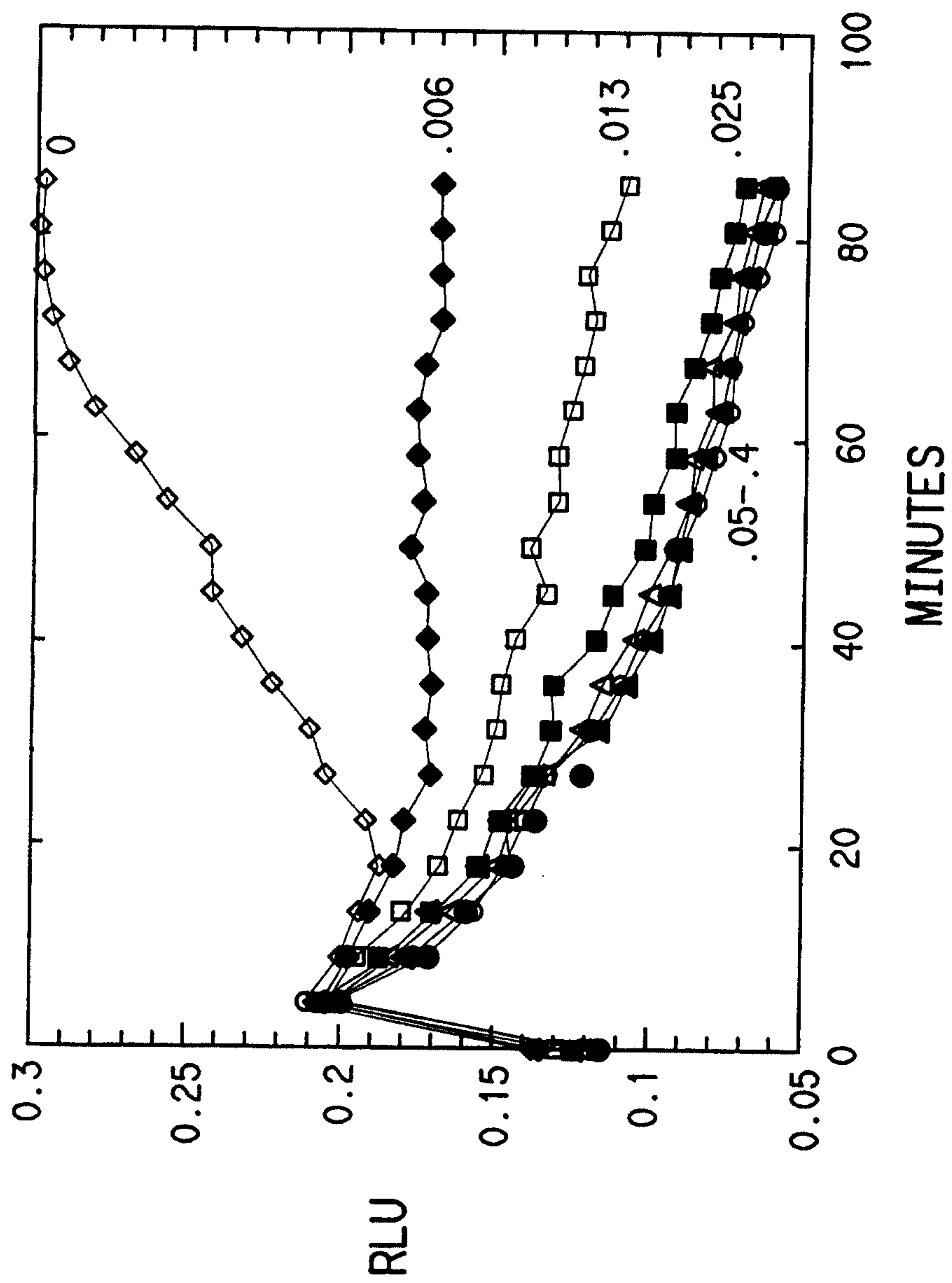


FIG. 10B

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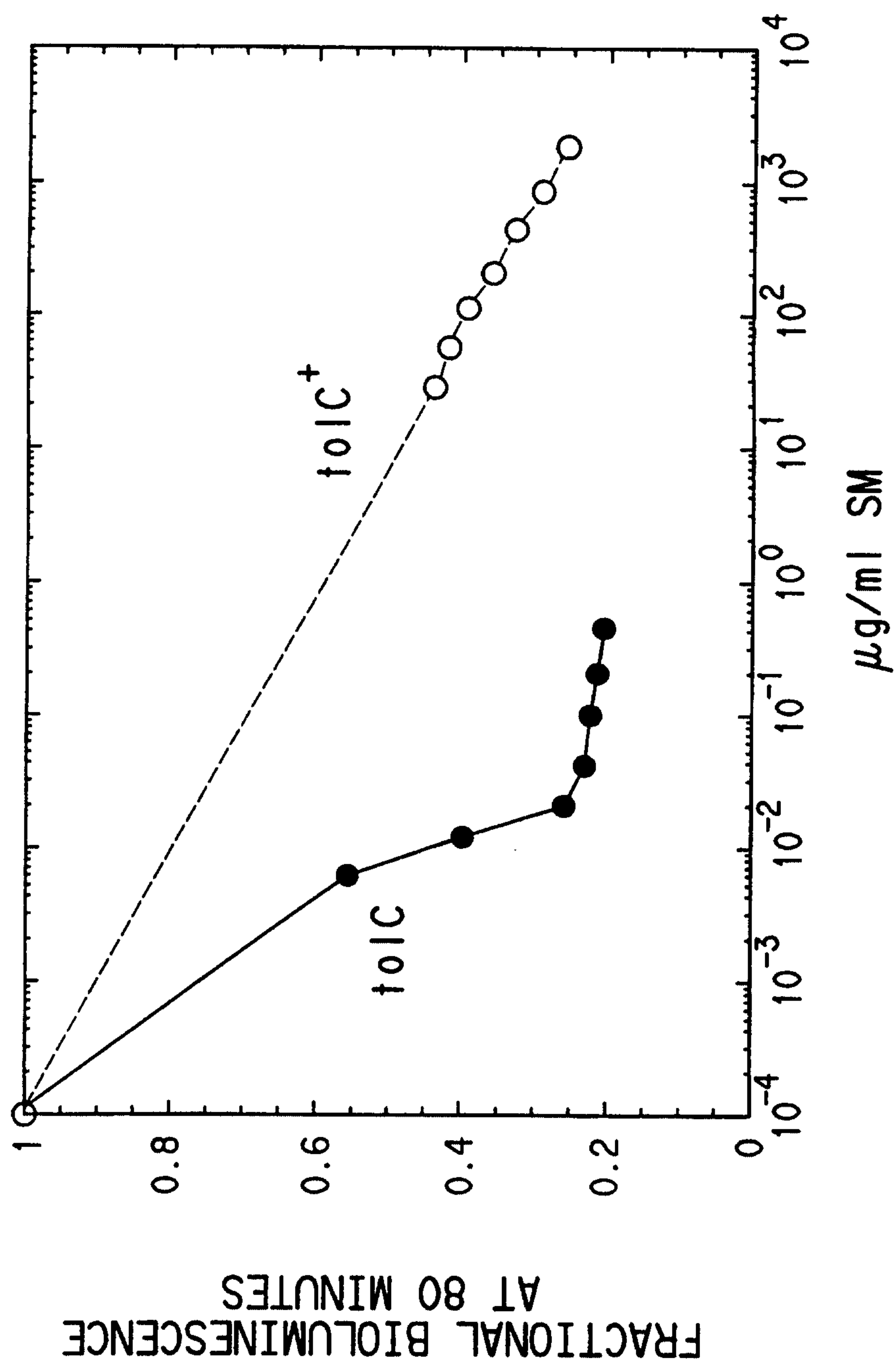


FIG. 10C

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○ A3/4-100 μ g/ml
● B3/4-50 μ g/ml
▲ C3/4-25 μ g/ml
△ D3/4-12.5 μ g/ml
■ E3/4-6.25 μ g/ml
□ F3/4-3.12 μ g/ml
◆ G3/4-1.56 μ g/ml
◇ H3/4-no add
— A11/12-DMSO

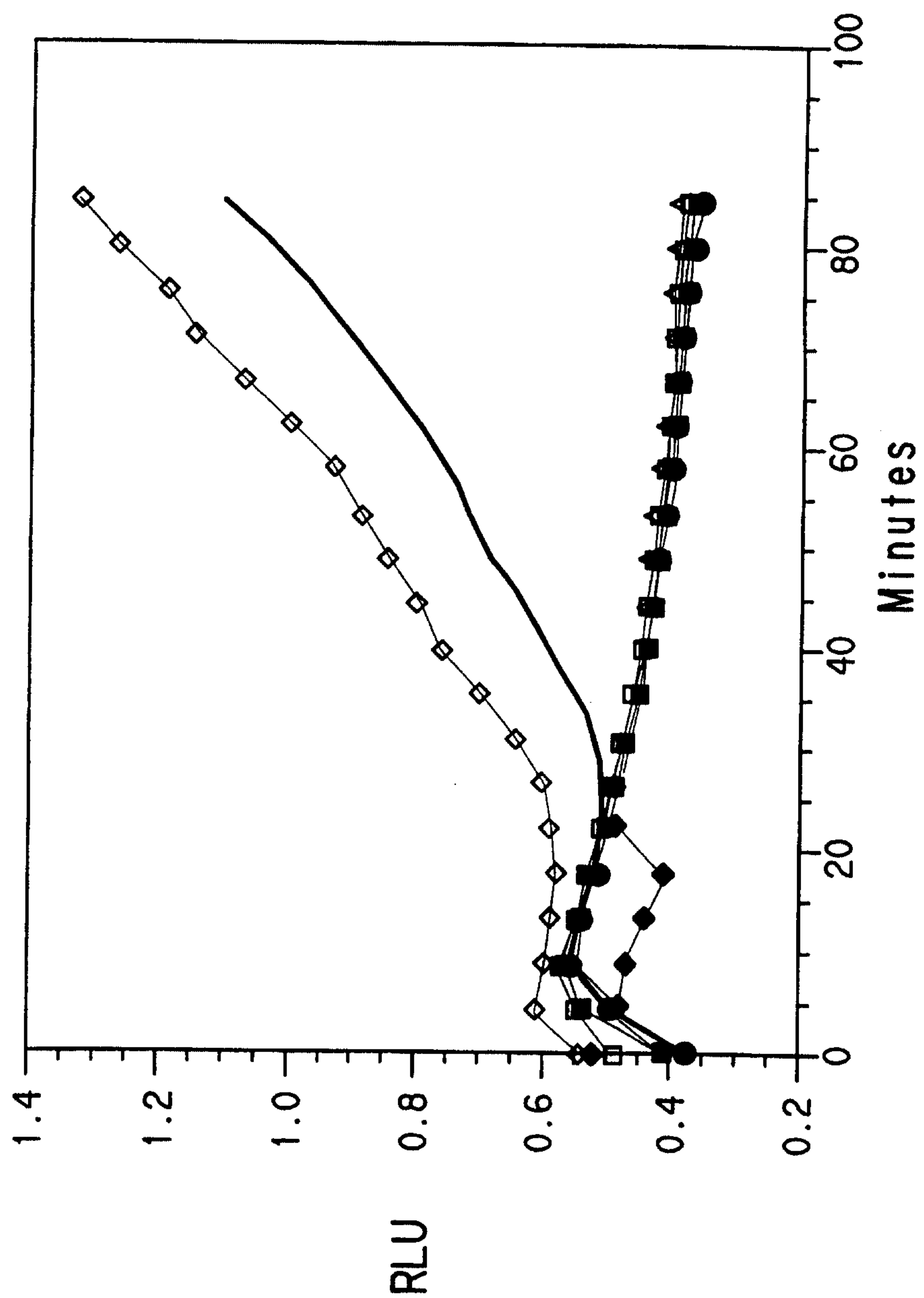


FIG. 11A

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A11/12-DMSO
 A5/6-100 μ g/ml
 B5/6-50 μ g/ml
 C5/6-25 μ g/ml
 D5/6-12 μ g/ml
 E5/6-6 μ g/ml
 F5/6-3 μ g/ml
 G5/6-1.56 μ g/ml
 H5/6-no add

○ ● ▲ △ ■ □ ◆ ◇ *

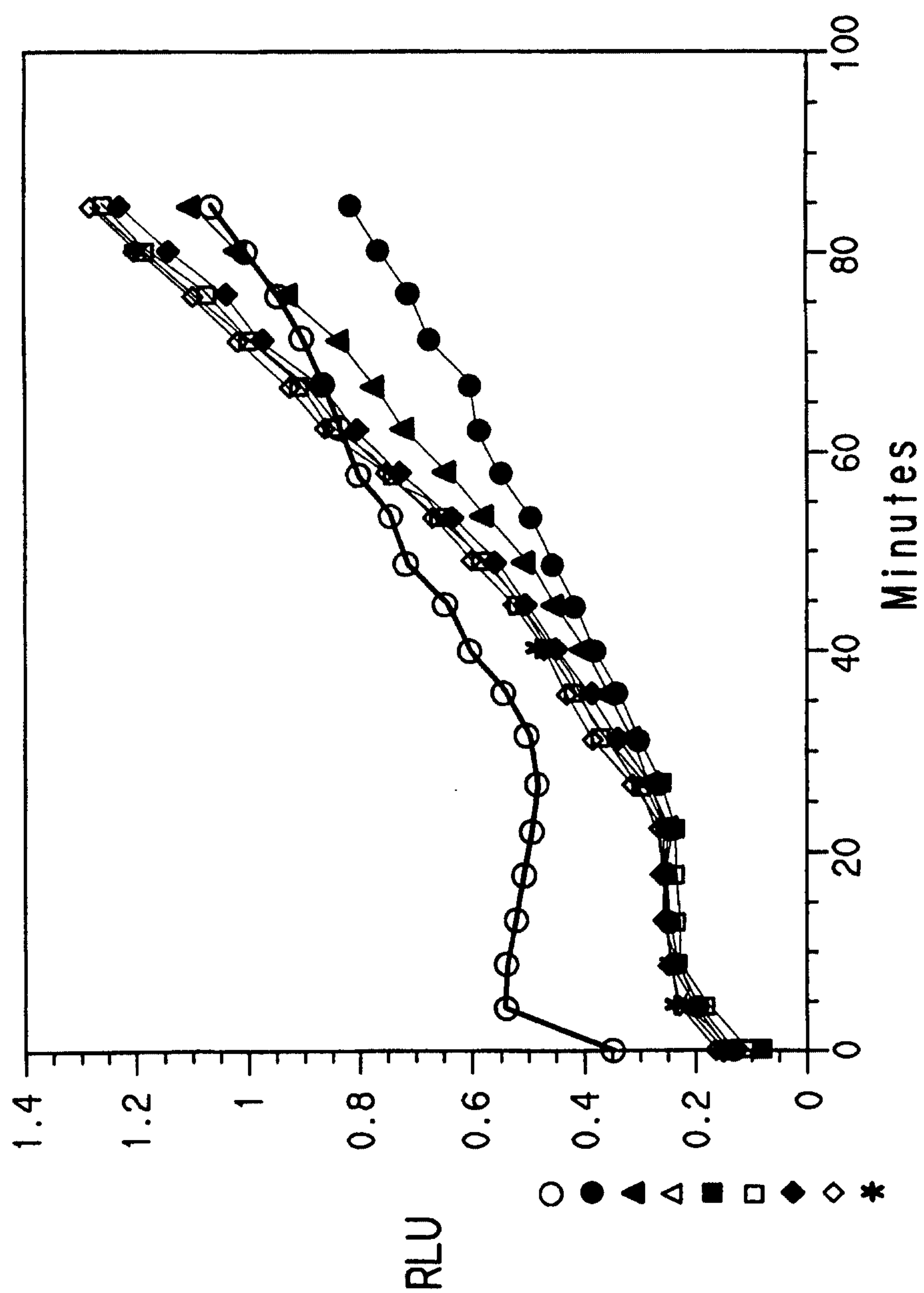


FIG. 11B

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○ A1/2-100 μ g/ml
● B1/2-50
◆ C1/2-25
△ D1/2-12.5
■ E1/2-6.25
□ F1/2-3.1
◆ G1/2-1.56
◇ H1/2-no add

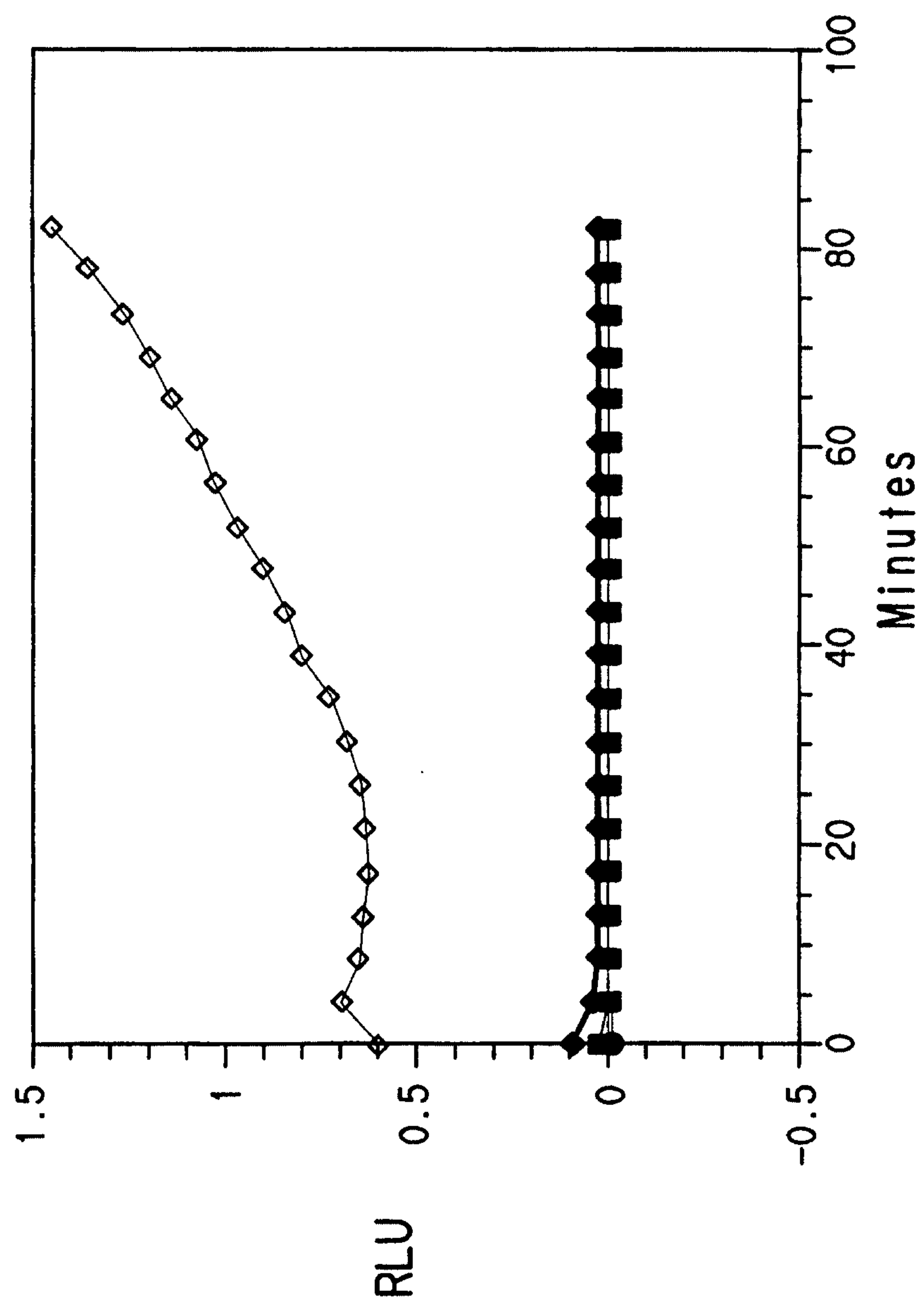


FIG. 12A

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○ A9/10-1000 $\mu\text{g/ml}$
● B9/10-500
▲ C9/10-250
◆ D9/10-125
■ E9/10-62.5
□ F9/10-31.25
◆ G9/10-15.6
◇ H9/10-no add

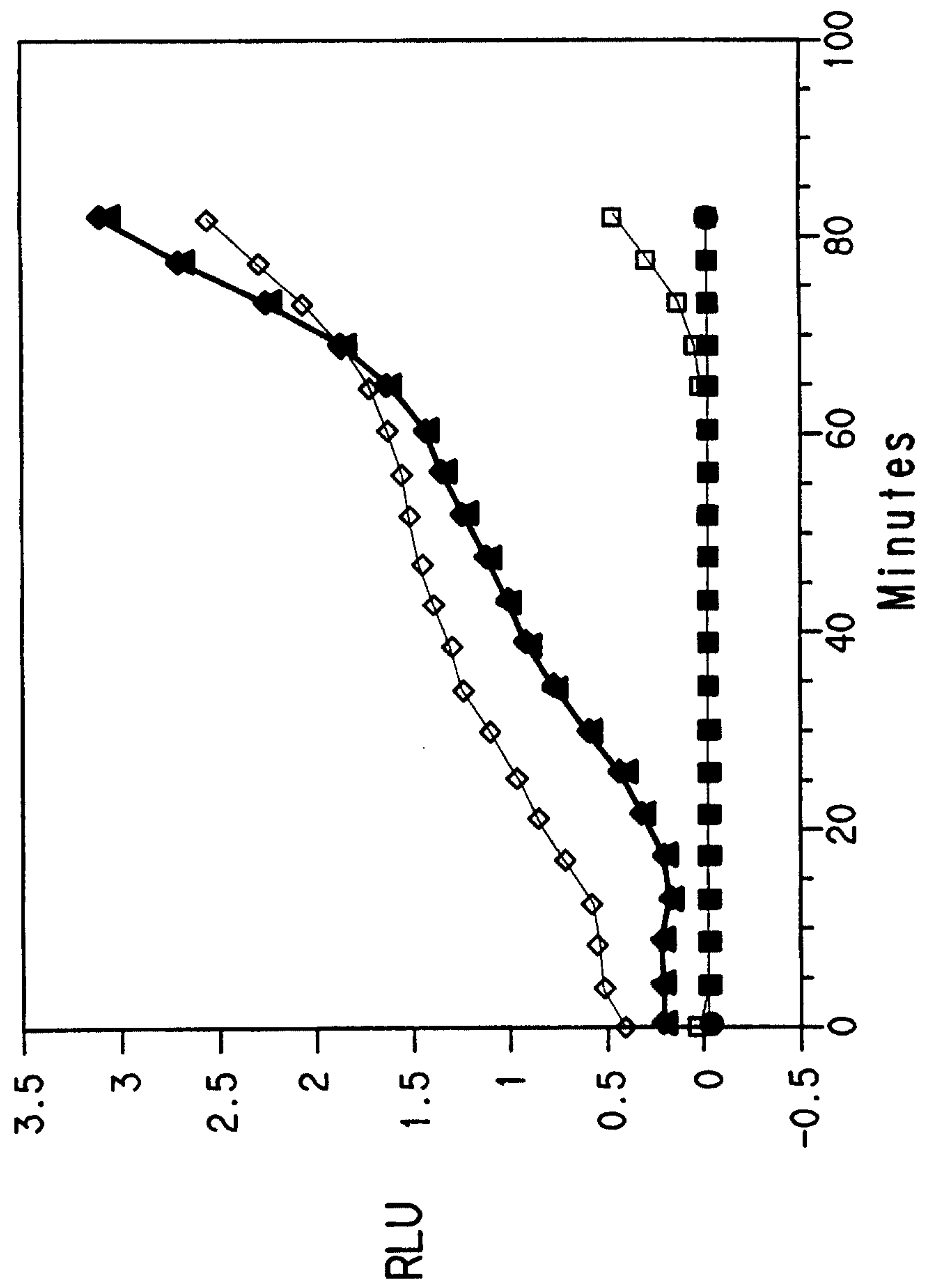


FIG. 12B

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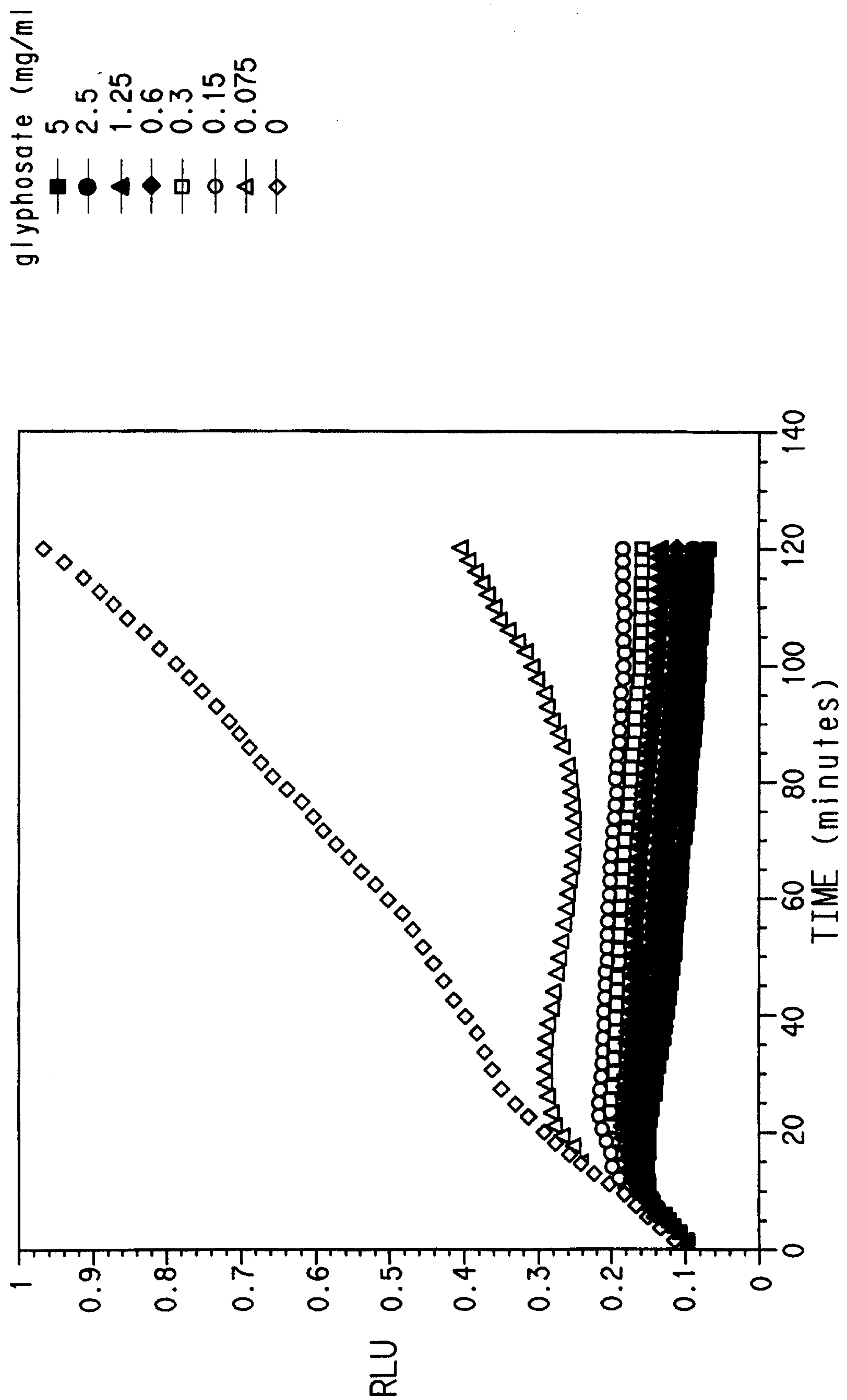


FIG. 13A

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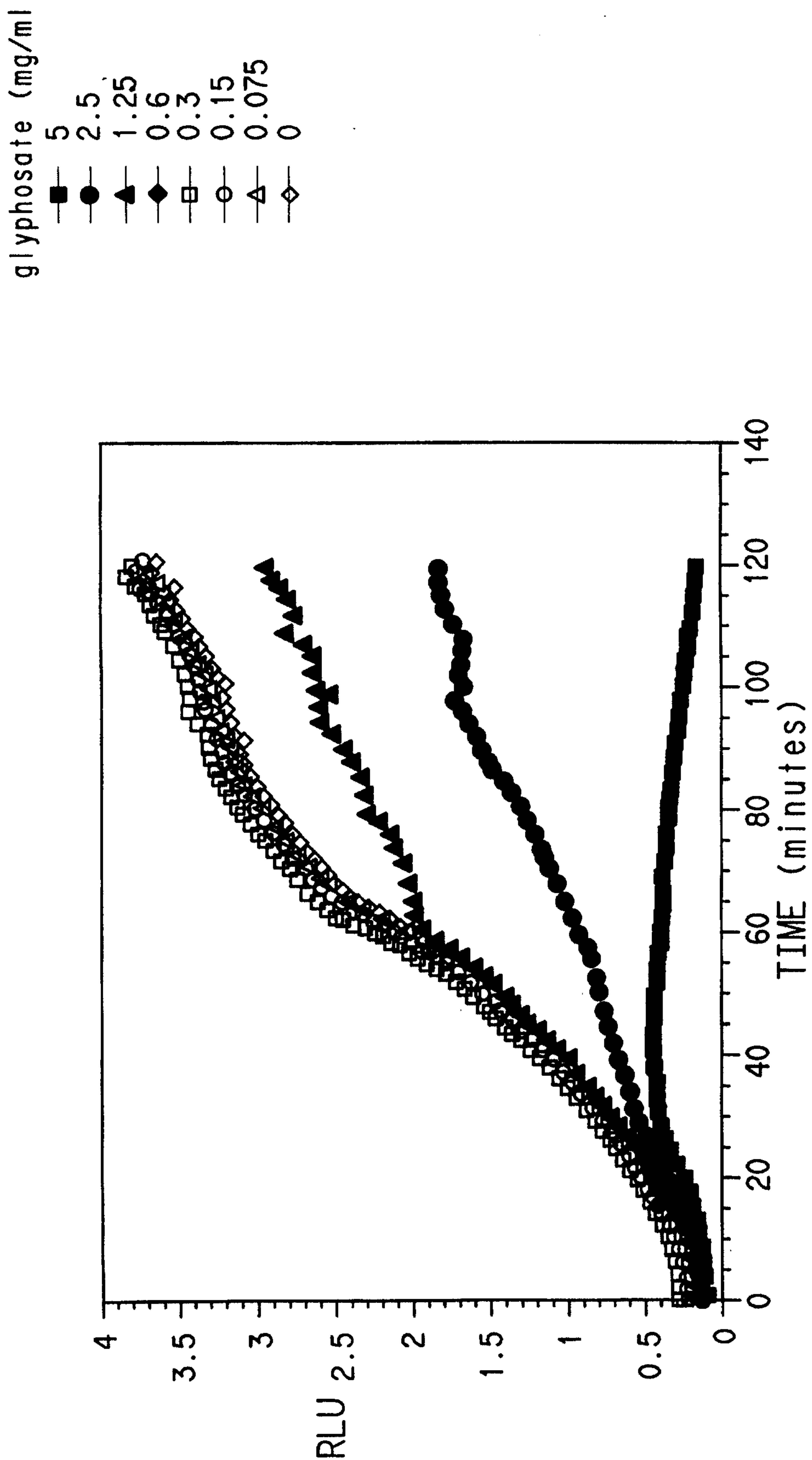


FIG. 13B

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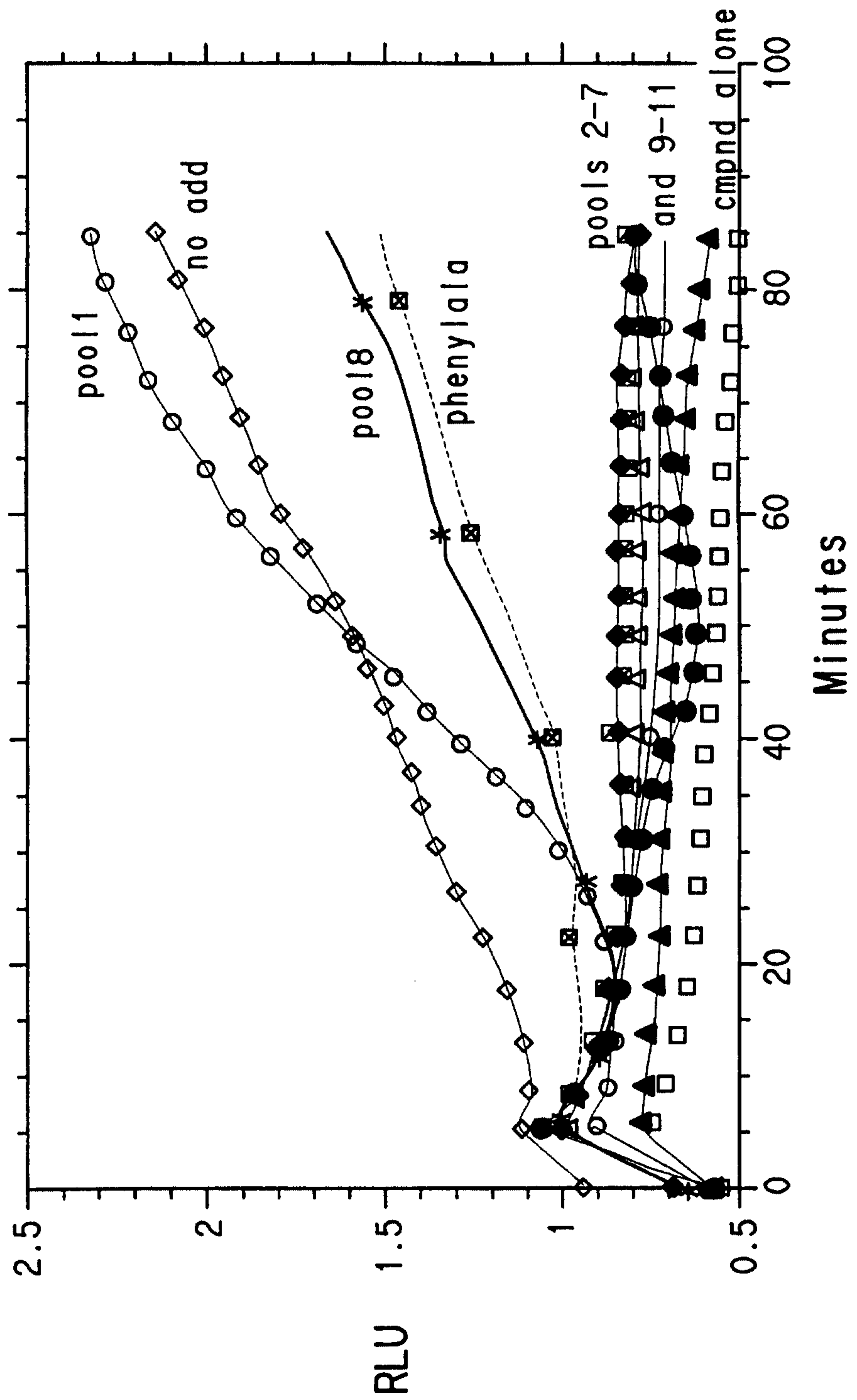


FIG. 14

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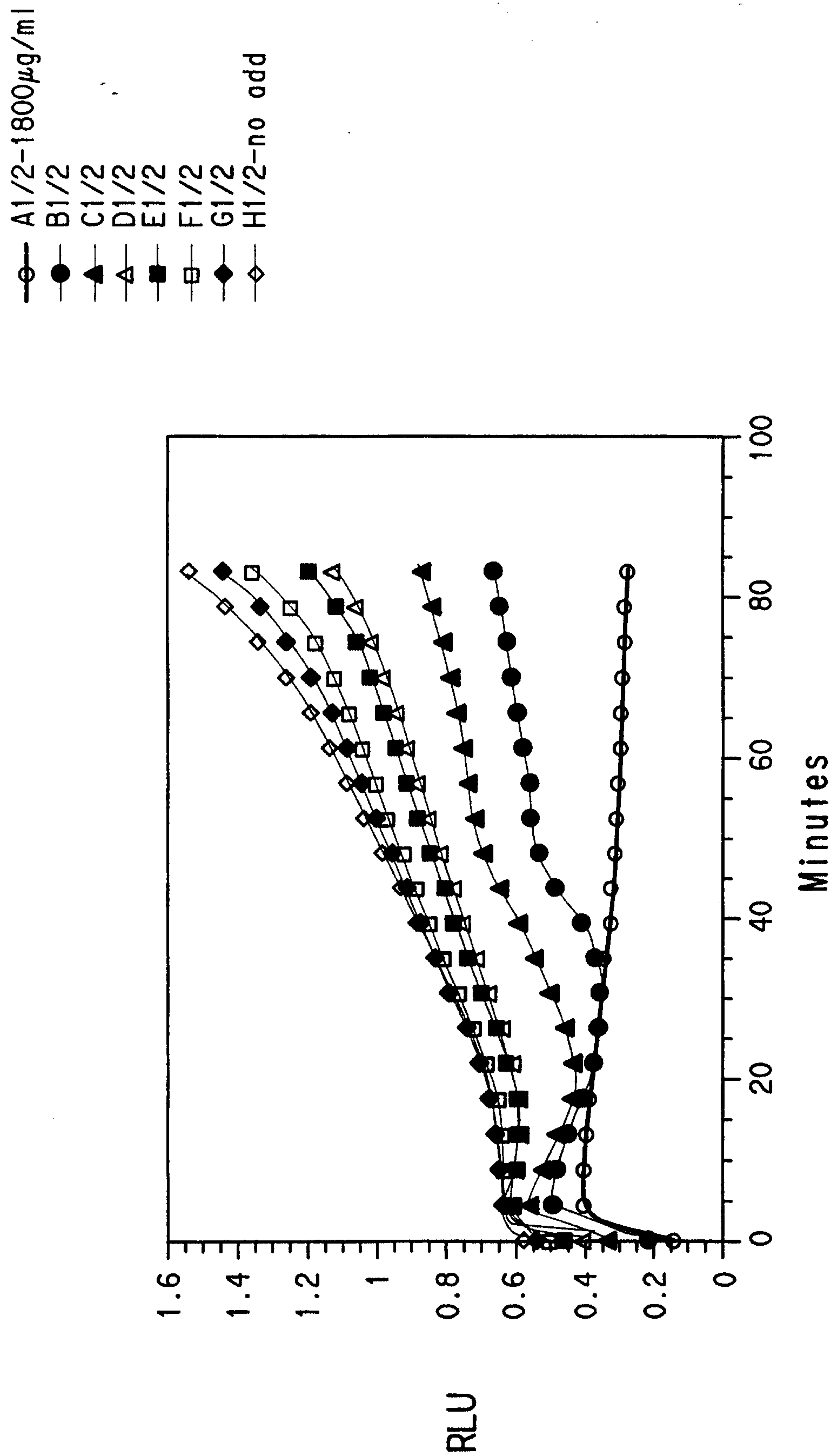


FIG. 15A

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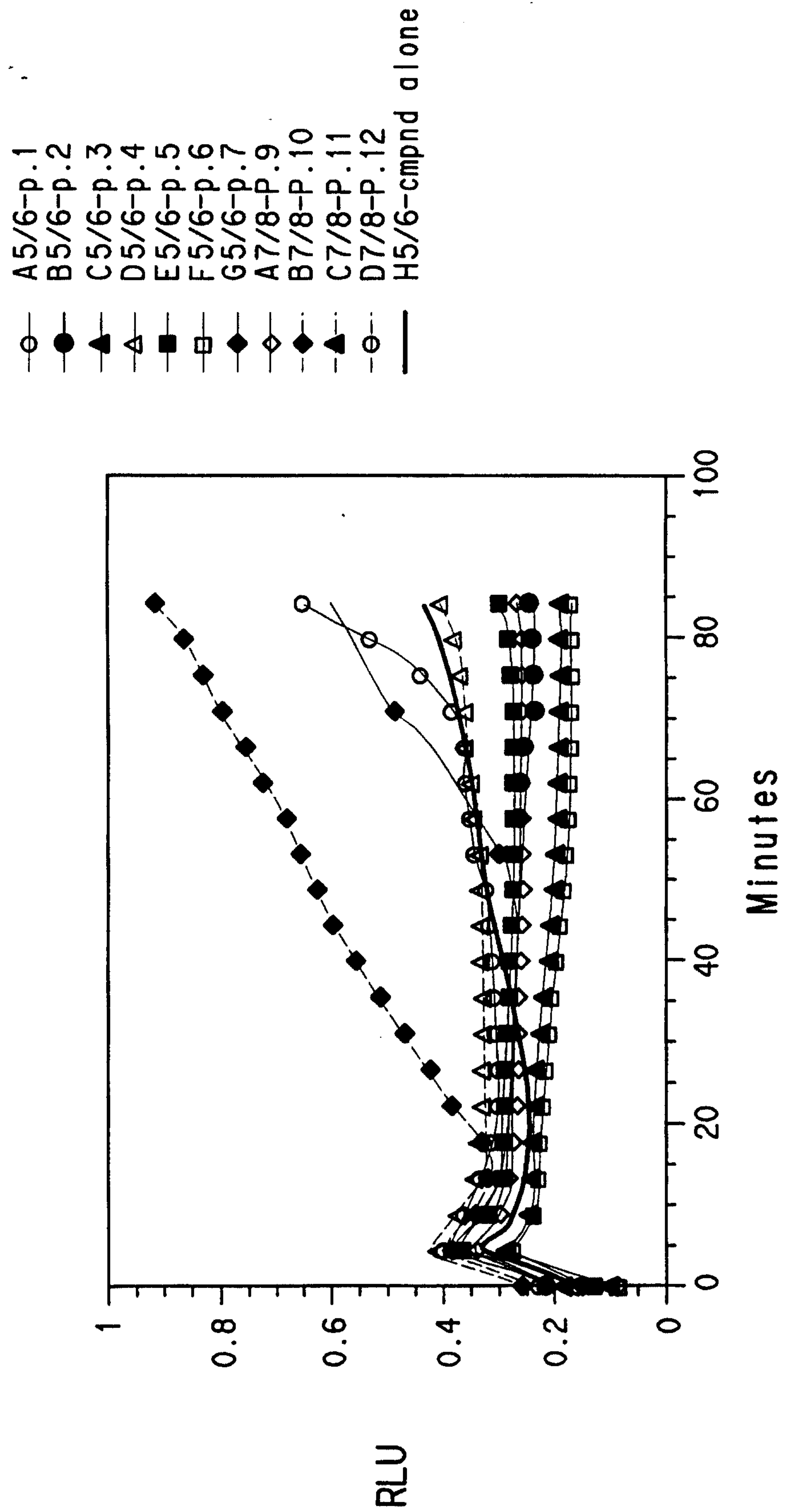


FIG. 15B

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