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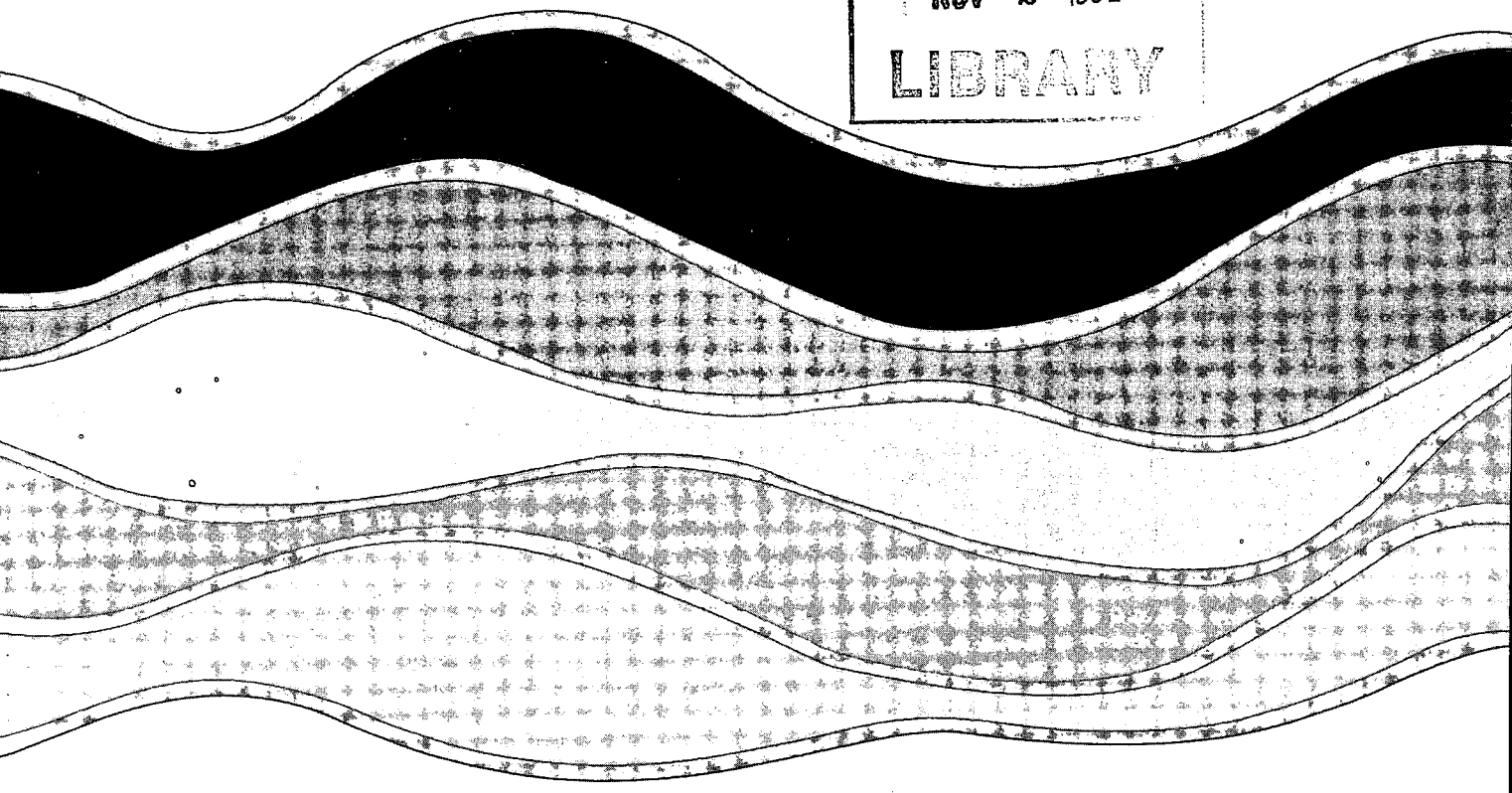

TUBE BASED ENZYME-IMMUNOASSAY FOR ATRAZINE: METHOD DESCRIPTION

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NWRI Contribution No. 92-14

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MANAGEMENT PERSPECTIVE

It is important that pesticide levels be monitored in aquatic ecosystems. In recent years the use of alternative herbicides, such as the s-triazines, has increased. Conventional methods for the determination of s-triazines, such as atrazine, in environmental samples are tedious and cost over $100 a sample. Screening tests that could eliminate samples that are either atrazine free or contain less than a threshold level of the herbicide, would improve the efficiency of analytical laboratories. The modified enzyme-immunoassay (EIA) for atrazine satisfies the requirement for such a screening capability.

The EIA for atrazine can be used to screen large sample sets for the presence of atrazine and a broad range of other triazine herbicides. The EIA can also be used to monitor atrazine levels in waters that are known to be contaminated with that herbicide, and to rapidly confirm the results of conventional analyses. The EIA is suited to field use where it can help to identify contaminated sites.

SOMMAIRE À L'INTENTION DE LA DIRECTION

Il est important que les teneurs en pesticide soient surveillées dans les écosystèmes aquatiques. Au cours des dernières années, l'utilisation d'herbicides de remplacement comme les s-triazines a augmenté. Les méthodes habituelles pour le dosage des s-triazines, p. ex. l'atrazine, dans les échantillons environnementaux sont compliquées et coûtent plus de 100 $ par échantillon. Des essais de dépistage permettant de déterminer si des échantillons sont exempts ou non d'atrazine (pour une valeur seuil donnée) permettraient d'améliorer l'efficacité des laboratoires d'analyse. L'immunoessai enzymatique (IEE) pour l'atrazine satisfait aux exigences d'un tel dépistage.

L'IEE pour l'atrazine est utilisé pour déceler la présence d'atrazine dans d'importants ensembles d'échantillons, ainsi que pour une vaste plage d'autres herbicides de type triazine. L'IEE peut être utilisé pour surveiller les concentrations dans les eaux dont on sait qu'elles sont contaminées par cet herbicide, et pour confirmer rapidement le résultat des analyses habituelles. L'IEE est bien adapté aux utilisations sur le terrain, et il peut faciliter l'identification des emplacements contaminés.

ABSTRACT

The enzyme-immunoassay (EIA) for the detection of atrazine and other triazine herbicides can improve analytical efficiency through the identification of triazine free samples and samples that contain less than a threshold level of the herbicide. The present report describes a modified version of a tube based EIA for the detection of triazine herbicides. The assay is commercially available. The assay is repeatable, rapid (30 min), and sensitive (the estimated error associated with zero dose is 22 pg). Although the assay can be used in the laboratory, it is more suited to field use. The report is intended as an aid for analysts who wish to implement the technique.

RÉSUMÉ

L'immunoessai pour la détection de l'atrazine (IEE) et d'autres herbicides à base de triazine peut améliorer l'efficacité des analyses par l'identification des échantillons exempts de triazine et des échantillons contenant moins qu'une certaine valeur seuil de cet herbicide.

Ce rapport décrit une version modifiée d'un IEE dont l'emploi se fait dans un tube pour la détection des herbicides de type triazine. Ces tubes sont disponibles sur le marché. Il s'agit d'un essai répétable, rapide (30 min), et sensible (l'erreur estimée associée à une dose zéro est de 22 pg). Bien que cet essai puisse être utilisé en laboratoire, il est mieux adapté aux utilisations sur le terrain. Ce rapport est destiné à faciliter le travail des spécialistes de l'analyse qui veulent mettre en oeuvre cette technique.

INTRODUCTION

Atrazine is an s-triazine herbicide that is widely used for the pre and post emergence control of annual weeds in a variety of crops that includes corn (maize) and asparagus (1). Atrazine is also used non-selectively in crop free areas. Other s-triazine herbicides such as simazine and propazine are also popular for the control of broad leaf and monocot. weeds. Consequently there is a need for information on the distribution, pathways, and fate of the triazine herbicides in aquatic ecosystems. Conventional methods for the determination of triazines in water tend to be time consuming and expensive. The more traditional methods rely on solvent extraction followed by enrichment on a Florisil column (2). The analyte can be quantified by gas chromatography combined with either a nitrogen-phosphorous detector (GC-NPD) (2) or a mass spectrometer (3) (GC-MS).

Screening tests could improve analytical efficiency by facilitating the removal of atrazine free samples from the analytical process. Immunoassay (IA) screening techniques are widely used in clinical laboratories and are now being seriously considered for use in environmental applications. IAs are cost effective, rapid, and permit the simultaneous analysis of multiple samples (4 - 6). IAs have been developed for the detection of a wide range of pollutants including polychlorinated dibenzo-p-dioxins (7, 8), polychlorinated biphenyls (9), dieldrin and aldrin (10), molinate (11), 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) (12), clomazone (13, 14) and alachlor (15). The relevance of immuno-technologies to environmental chemistry has been recently reviewed (29).

Several EIAs that have been developed for the detection of the s-triazine herbicides and their degradation products in water (17 - 23) could prove useful for monitoring atrazine levels in surface and ground waters. However, in order to be of use, the antibodies, on which the IAs are based, must be readily available. ImmunoSystems

Inc. (IMS, Maine, USA) has pioneered the commercial distribution of IAs for environmental contaminants. IMS have developed and marketed a tube based EIA for the detection of triazine herbicides (24).

This present report is a detailed description of a version of IMS's EIA procedure that has proven successful in our laboratory. It is intended to assist analysts who may wish to implement the method in their laboratories. A full validation of the assay has been described in an earlier report (29). In brief, the assay could detect atrazine in lake and river water with detection limits of 62 pg/mL and 180 pg/mL respectively. The assay's ability to quantify atrazine in a set of 124 water samples taken from many parts of Canada was compared with a GC-NPD reference method (R=0.919). A 71% reduction in analytical load was reported (29) at a threshold concentration of 1 ng/mL. There were 2.4% false negative and 0.8% false positive results associated with that load reduction. The variability of the assay control parameters was generally within two standard deviations of the mean response for 65 assays.

The immunoassay for atrazine is fast (30 min), sensitive (22 pg), and has a low detection limit (67 pg). The method is cost effective ($20 per sample), requires minimal sample preparation (pH adjustment), and is easily learnt. Analysts can use the EIA to screen large sample sets for the presence of atrazine and other triazine herbicides. The EIA can also be used to monitor waters that are known to be contaminated with atrazine, and to rapidly confirm the results of GC-NPD analyses. The EIA is ideal for field use where it can help in the identification of contaminated sites.

Scope and Application

The EIA for atrazine cross reacts with several triazine herbicides and related compounds: prometryn 100%[1], dipropetryn 100%, propazine 80%, ametryn 57%,

[1] Cross reactivity (%) with respect to atrazine.

cyprazine 57%, prometon 57%, atratone 40%, simazine 16%, simetryn 16%, terbuthylazine 10%, cyanazine 1%, trietazine 4%, 6-hydroxy atrazine 1.4%, de-ethylated atrazine 4%, and terbutryn 2.7 %. Several herbicides such as alachlor, 2,4-D, and glyphosate, that are likely to be found in association with atrazine do not cross-react in the assay. The ability of the assay to detect a broad range of s-triazine herbicides is not a problem since the assay can be used as a screen for the triazines as a herbicide class. The EIA can produce semi-quantified data for any of the cross-reacting herbicides if they are present as the dominant triazine. As a general rule, samples that test positive in the EIA should be confirmed by an independent technique.

The present version of the EIA is suitable for use with water samples. No extraction or clean-up of the water samples is required. The EIA for atrazine should be applicable to solid environmental matrices, provided that a suitable extraction procedure is used. The type of clean-up procedure would have to be established for each matrix type. Nevertheless, it is anticipated that minimal clean-up would be required; in most cases the EIA should require far less clean-up than conventional techniques.

The EIA permits the rapid semi-quantitative analysis of water samples. This advantage should be exploited to the full. It is no longer necessary for laboratories to spend appreciable time processing largely negative sample sets. Residue laboratories can now lower the unit cost of atrazine determinations and/or process more samples in a given time period. Moreover, the EIA enables clients to eliminate negative samples before submission to the analyst.

Principle and Theory

Based on the classical antigen - antibody reaction (25), IAs are relatively simple, powerful, and adaptable techniques for the rapid determination of trace levels of organic compounds. Originally developed for the micro-determination of proteinaceous substances (26), IAs are also extensively used to detect and determine steroidal hormones,

antigens, drugs, and other low molecular weight organic molecules. Immunochemical methods, while being traditionally unfamiliar to the residue chemist, offer exciting possibilities for newer cost effective approaches (27).

The EIA for atrazine is a direct competitive assay. The anti-atrazine antibodies are immobilized on test tube walls. The analyte and an atrazine tracer that is labelled with a peroxidase enzyme are added to the tube and allowed to incubate. After a short period the bound and free tracer molecules are separated by a simple washing technique. The bound enzyme is quantified by its ability to convert a substrate. The enzyme's activity causes a colour change in the tubes which can be measured by a spectrophotometer. A series of atrazine standards is used to prepare an assay calibration curve. The amount of bound enzyme activity is normalized with respect to an analyte free reference tube (B_0) and then plotted against the amount of atrazine on semi-log graph paper. A reduction in the amount of bound enzyme is inversely proportional to the quantity of analyte present in the assay tube. Analyte concentration is interpolated from the standard curve. A sample is considered positive if it is distinguishable from a matrix blank using Student's t test (95% confidence interval).

Assay sensitivity is defined as the precision of measurement of zero dose. It is estimated from the standard deviation of the analyte free reference tubes. The assay detection limit is considered in two ways: the minimum detectable concentration (MDC) and the lowest concentration detected (LCD). The former is a deduced estimate of the DL based on statistical criteria (3 x the error associated with zero dose), whereas the latter is an empirical observation (the lowest concentration that was measured in practice).

The EIA for atrazine has been successfully used to eliminate negative samples at a threshold concentration of 1 ppb (16). Used as a screening test the EIA facilitated a 71% reduction in the sample load for the analytical laboratory. That load reduction was achieved at a cost of 2.4 % and 0.8 % false negative and false positive results

respectively. At a lower threshold level of 0.5 ppb the reduction in sample load was 64 % and the false negative and positive results would be 2.4 % and 4 % respectively.

Interferences

The only compounds known to cause false positive results in the assay are other triazine herbicides. Samples containing such interferences would probably also be of interest to the analyst. Substances in a water sample that can denature antibodies will cause false positive results. Although not a common event, it has been our experience that very low pH values can cause such an effect. As a precaution the pH of samples should be checked prior to analysis by EIA. If necessary the pH of the sample can be adjusted with a small portion (0.1 mL to 0.9 mL) of PBS (10 X) and NaOH or HCl. If heavy metals are a problem some EDTA (1.9 g/100 mL) can be incorporated in the PBS.

Based on our experience the EIA for atrazine appears to be robust. However that is no guarantee that the assay will be problem free with all water types. The experienced analyst must be relied upon to devise simple solutions for any problems that may arise.

Apparatus

Water bath:

An ambient assay temperature of 25 °C is maintained using a constant temperature bath (Blue M, Magni Whirl) that is fitted with a shaker motor.

Plate reader:

A Bio-Tek plate reader (Model EL 312) is used to read the optical density (OD) of the final assay solution at 450 nm; the plate reader is programmed to take a dual wave length reading at 615 nm and at 450 nm; the 615 nm value is automatically subtracted from the 450 nm one so as to compensate for imperfections in the plastic. The data are transferred to a computer (Olivetti XP1056) via the an Elisar software utility (28) where they are processed in a spreadsheet (VPP3D, Paper Back Software). The data reduction templates can be made available as part of a technology transfer process.

Glassware:

Hamilton micro-syringes to cover the range 10-500 μL.
glass tubes with teflon lined screw caps (for atrazine standards).
glass beakers of miscellaneous sizes.
pasteur pipettes.
Micro-titre plates.

Additional equipment:

4 °C incubator.
Vortex mixer.
Eppendorf adjustable pipettes.
Positive displacement pipettors that can accommodate glass tips.
Tube racks.
Timer.
Wash bottle.

REAGENTS

Assay Kits

The Res-I-Mune kits for the detection of triazine herbicides can be purchased from ImmunoSystems Inc. (Maine, USA). The kits should be stored at 4 °C.

Standards

An atrazine (Chem Services/Mandel Scientific) stock solution is prepared in methanol (200 mg/L). Intermediate stocks and working solutions are prepared by dilution in Milli-Q water. It is recommended that the working stocks be prepared using a linear dilution series. Each new set of stocks should be checked for accuracy by comparison with a reference stock. Fresh working stocks should be prepared on a regular basis, or if a deterioration in the assay's response is observed. Although the working stock solutions can also be prepared by serial dilution from a master stock, such procedures are susceptible to systematic dilution errors which can cause curve shifts and inter assay drift.

Other reagents:

Deionised water: Milli-Q. Beware! The EIA has detected atrazine in Burlington tap water.

Methanol: pesticide grade.

Kit Standardization:

Based on our experience (16) the assay procedure should be fine tuned for each batch of kits. The kits should be coded upon receipt and the B_0 response for a tube from each kit should be determined and recorded in a log book. The following assay protocol can be used to determine the B_0 response. If the B_0 response is much different from 1.0 units, the duration of the second incubation step (enzyme - substrate) can be adjusted. Tubes and reagents should not be shared between kits as a precaution against inter-kit variability.

Assay Protocol:

- Bring the reagents, assay tubes, standard solutions, and water samples to room temperature.
- Transfer the enzyme conjugate, substrate, and chromogen reagents to pre-labelled tubes. Stopper the tubes and place them in the water bath to equilibrate.
- Place the water samples, atrazine standards and some Milli-Q water in the water bath to equilibrate.
- Label the assay tubes. Usually ten tubes are processed at a time. Tubes 1 and 10 are antigen free (B_0), tubes 2-4 contain known amounts of atrazine, and tubes 5-9 contain either additional standards, or water samples.

- Add 160 μL of Milli-Q water to the reference tubes. Add 160 μL of the appropriate atrazine standards (0.1 or 0.2 ppb, 1 ppb, and 10 ppb) to tubes 2-4. Add 160 μL of sample or quality control standard to the remaining tubes.
- Use a repeating pipettor (Eppendorf) to rapidly add enzyme tracer (150 μL) to each assay tube. Gently flick the tube and then immediately vortex mix it for 3 seconds.
- Incubate the tubes for 15 min.
- Decant the tubes. Use a wash bottle to fill them with Milli-Q water. Decant the water. Repeat the foregoing wash cycle 3 times. After the last wash the remaining water droplets are shaken from the tubes with a sharp flick. The tubes are then inverted and allowed to dry for 5-10 min. on an absorbent paper pad after which the tubes are returned to the water bath.
- Enzyme substrate (150 μL) followed immediately by chromogen reagent (150 μL) is then added to each tube. Promptly vortex mix each tube for 3 seconds after the addition of the latter reagents. Stagger the addition of the substrate and chromogen in order to equalize the enzyme reaction times.
- After 3 min. (the duration of this step should be optimised for each batch of kits) add a drop of stop solution (2.5 N H_2SO_4) to each of the tubes. Vortex mix each tube for 3 sec. The acid halts the enzyme reaction and changes the colour of the reaction mixture to straw yellow.
- Add 500 μL of Milli-Q water to each tube. Remix the tubes. Transfer three 200 μL aliquots of each reaction mixture a 96 well micro-titre plate.
- Use the Bio-Tek plate reader to determine the OD of the reaction mixtures.
- Return any left over reagents to the 4 °C incubator.

Data Reduction and Analysis:

The normalized assay response is defined as the mean optical density of the replicate standard or unknown wells (B) divided by the optical density of the reference tubes (B_0) and multiplied by 100.

- Determine the mean OD of each set of replicate wells.
- Plot (Bound (B)/Reference (B_0)) x 100 versus Log_{10}[atrazine] for each standard. A four parameter logistic function or a polynomial function can be used to fit a curve to the plotted data. Jandel Scientific's TableCurve software package is recommended for this purpose. Particular care should be taken to ensure that any polynomial expressions used are well behaved at the extremities of the calibration curve.
- Estimate the samples' atrazine content from the calibration curve.
- Express the data as atrazine equivalents.
- Several assay trend parameters can be used to monitor the EIA's performance. The following parameters are recommended: B_0, 0.1 ppb standard, and 1 ppb standard. An additional quality control (QC) standard can also be included. The QC standard should be prepared from a different stock solution than the assay standards, preferably using an independent dilution protocol.
- Some trouble shooting will be required if any of the control parameters deviate by more than 2 x SD from the mean response or if evidence of inter assay drift is apparent from the plots. Firstly, verify the integrity of the standards: use the 1 ppb standard that is supplied with the kit as a reference. The second incubation period should be re-optimised if the B_0 response has drifted.

Threshold Screen:

There will inevitably be some border line samples that fall close to the cut-off value. Such samples should be re-analyzed, preferably with replication. Any obviously dubious data should also be re-analyzed. Examples of such data include low or high values within groups of samples that have predominantly high or low atrazine contents. As with any technique the EIA demands a conscientious operator who will stay on top of the data. Given such an attitude the method can produce valuable and reliable data.

Operation Notes and Considerations:

Samples should not be spiked with an internal standard as it will be detected by the antibodies.

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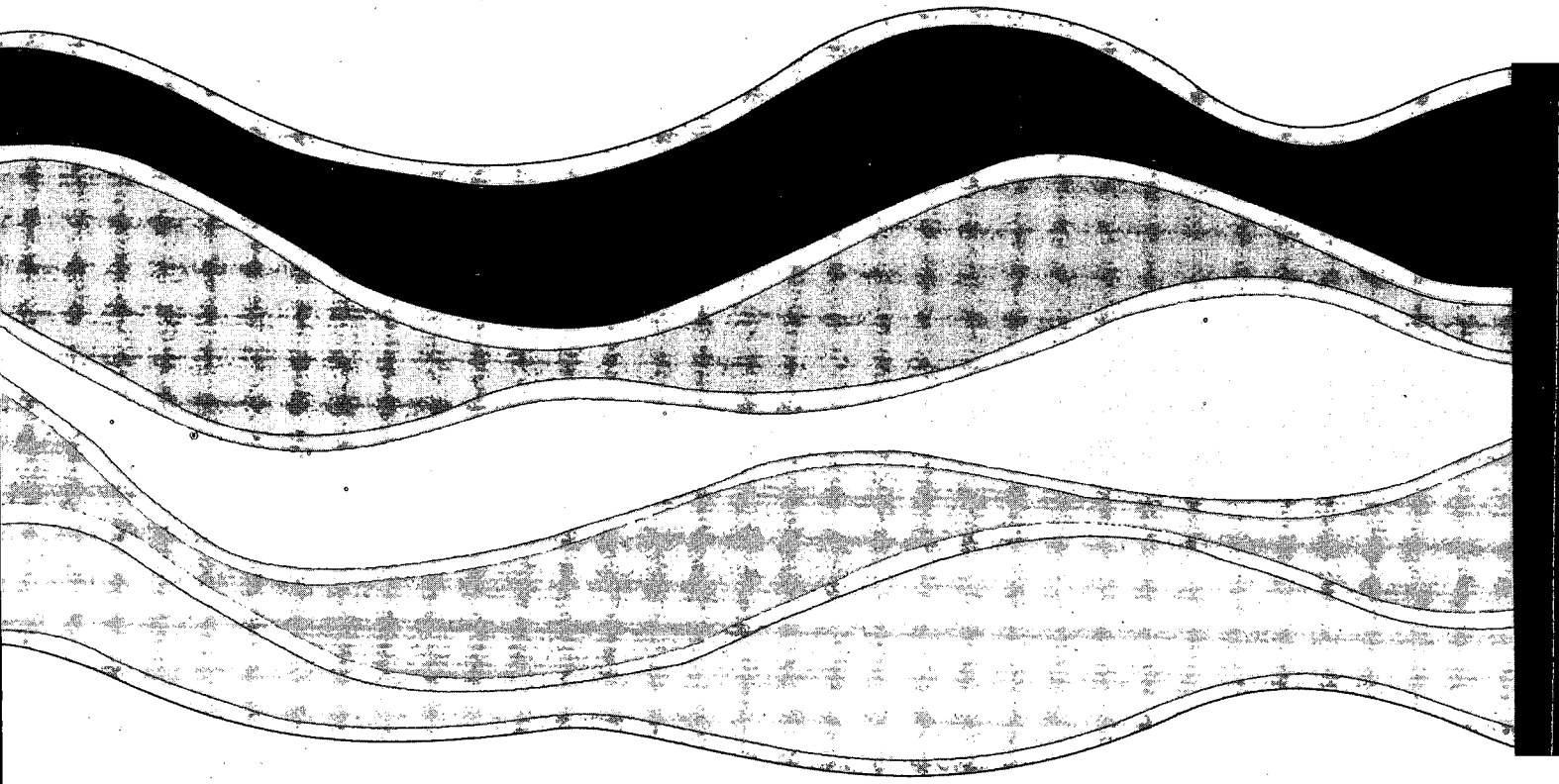

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