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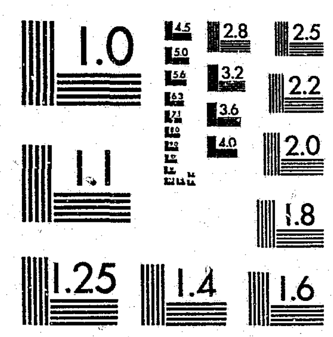

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U. S. DEPARTMENT OF JUSTICE
LAW ENFORCEMENT ASSISTANCE ADMINISTRATION

CATEGORICAL GRANT PROGRESS REPORT

GRANTEE	LEAA GRANT NO.	DATE OF REPORT	REPORT NO.
John Jay College / CUNY Research Foundation, City University of NY	79-NI-AX-0120	Dec. 22, 1982	10

IMPLEMENTING SUBGRANTEE

TYPE OF REPORT
☐ REGULAR ☐ SPECIAL REQUEST
☒ FINAL REPORT

SHORT TITLE OF PROJECT
Novel Tagged Antibody Method for Bloodstains

GRANT AMOUNT
$48,677

REPORT IS SUBMITTED FOR THE PERIOD Oct. 1, 1979 THROUGH Sep. 30, 1982

SIGNATURE OF PROJECT DIRECTOR

TYPED NAME & TITLE OF PROJECT DIRECTOR
Peter R. De Forest, Prof. of Criminalistics

COMMENTS (REPORT HERE (Add continuation pages as required.)

FINAL REPORT

(see attachment)

NOTE: *No further monies or other benefits may be paid out under this program unless this report is completed and filed as required by existing law and regulations (FMC 74-7; Omnibus Crime Control Act of 1976).*

RECEIVED BY GRANTEE STATE PLANNING AGENCY (Official)	DATE

LEAA FORM 4587/1 (REV. 2-77) REPLACES EDITION OF 10-75 WHICH IS OBSOLETE.

Final Project Report

by

Peter R. De Forest and Robert Rothchild

Our overall project was a pilot study designed to develop and evaluate a novel approach to cellular antigen typing in dried bloodstains. While this study was confined to the ABO blood group system, the concept had the potential of being applied to other red blood cell (RBC) antigen systems as well. The long range goal was the development of applications to other antigen systems with the possibility of making all RBC antigen determinations simultaneously. The potential for use with semen or hair samples could also be an outgrowth of this work. Our approach consisted of several more or less distinct phases.

The first phase involved design and synthesis of taggant molecules. These taggants had to be capable of attachment to purified antibodies, with a different tag available for each different antibody. The uniquely labeled antibodies were to be applied to a sample of dried bloodstain, allowing homologous (labeled) antibodies to bind to blood antigens; unbound antibodies would be washed away. The washed sample would be treated to quantitatively release volatile fragments that would be chromatographically separable, permitting the quantitation and identification of the specific antibodies which had been bound.

The second phase involved the collection of bloodstains of different ABO groups and different ages, prepared on a

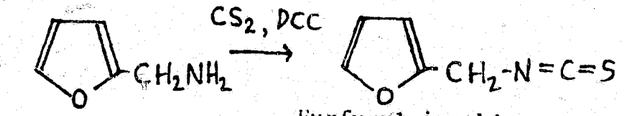
variety of substrates. The third phase dealt with the preparation and purification of immunoglobulin fractions. Antibody fractions of exceptionally high purity and specificity were sought to enhance the effectiveness of the method by minimizing interferences. The fourth phase dealt with the actual conjugation of the taggants to immunoglobulins and purification of the tagged antibodies. A fifth and final phase involves testing of labeled antibodies and method evaluation. Activity and specificity were to have been determined for the labeled antibodies and conditions optimized for the liberation of the tag molecule. A very substantial part of this project was to be concerned with the detailed elaboration and construction of modifications to a pyrolyzer and gas Chromatographic (gc) system and ancillary devices required for the actual liberation of tag molecules from samples and for the sensitive detection and determination of these tag molecules. This last aspect turned out to be one of the most crucial and demanding parts of the entire project.

Virtually all areas outlined above as part of our major project goals have been examined and largely completed. A summary of our successes and difficulties comprises this Final Report.

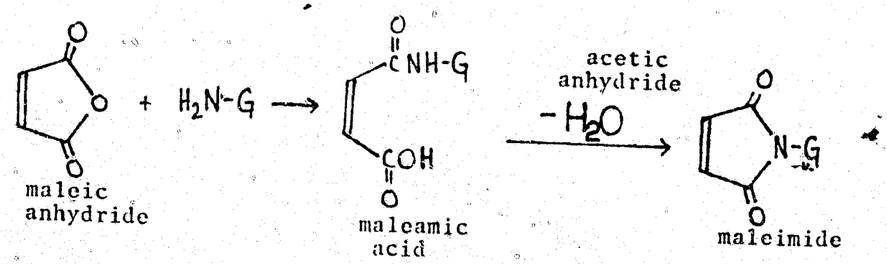
Phase I, the design and synthesis of taggant molecules: Examination of the literature led us in the direction of producing taggants that would be attached to ϵ-amino groups of antibody lysine residues by formation of a thiourea linkage via reaction at isothiocyanate groups. The method of liberation

of chromatographically separable, detectable and distinguishable "tags" was selected to be the mild pyrolysis of a Diels-Alder adduct. The detectable molecules, formed by pyrolysis and retro-Diels-Adler cleavage, were to be a series of N-substituted maleimides. Our target molecules were selected as being made up from the addition of furfuryl isothiocyanate to different maleimides.

These compounds were prepared as follows:



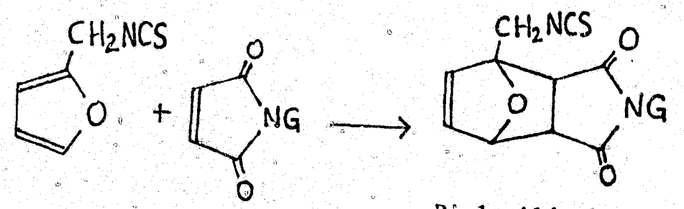
Furfuryl isothiocyanate



maleic anhydride

maleamic acid

maleimide



Diels-Alder adducts

An extensive series of maleimides was synthesized by the above scheme or purchased. Part of our concern was to develop a detection technique that would be exceptionally sensitive.

The maleimide structure was selected as being a good Diels-Alder dienophile (to permit adduct synthesis) as well as possessing good electron affinity. The latter feature should assure facile detection at low levels by means of a ^{63}Ni electron capture detector (ecd) on a gas chromatograph. Modifications of the group G would offer a simple means of synthesizing different labels for attachment to different antibodies; the different maleimides should be gas chromatographically separable with a silica capillary column. Some maleimides were commercially available and many others were synthesized. A number of these maleimides were previously unreported compounds. Some analogs were also examined, including maleic anhydride and some 2,3-dichloromaleimides. Although the latter promised enhanced dienophilicity and detection limits by ecd, these systems were evidently very labile. Reactions with these compounds evidently resulted in polymerization and formation of intractable tars. Characterization of synthesized maleamic acids, maleimides and Diels-Alder adducts with furfuryl isothiocyanate initially rested on infrared (ir) spectroscopy, mass spectrometry (ms) and ultraviolet (uv) spectroscopy. Much later, 60MHz proton nuclear magnetic resonance (^1H nmr) spectroscopy was carried out on these compounds following the acquisition of an nmr spectrometer through an NSF grant to the co-principal investigator, Dr. Robert Rothchild. Selected samples were also sent to outside analytical labs for elemental analysis. Ir of adducts showed anticipated absorptions of the isothiocyanate group and carbonyl resonances. Ms generally revealed molecular

ions (or metastable peaks consistent with the molecular ion) and, importantly, strong peaks at m/z values of furfuryl isothiocyanate and the corresponding maleimide. This was considered suggestive of a facile retro-Diels-Alder reaction occurring in the ms source and supported our belief that pyrolysis of bound, tagged antibodies would similarly release maleimides for gc detection. Correct elemental analyses were obtained for several representative adducts. With the subsequent availability of nmr for analysis of maleamic acids, maleimides and adducts, virtually all compounds were clearly shown to have spectra consistent with the assigned structures. Purity was generally good or at least adequate for our preliminary investigations. Many of the maleimides selected for study were N-phenyl maleimides with halogen atoms substituted on the benzene ring. These compounds held the promise of particularly high sensitivity detection by ecd.

At least six maleimides were shown to elute cleanly with reasonable retention times from a silica capillary column coated with a non-polar phase (methyl silicone) in a 50 meter length; at least six others were more extensively retained but, in most cases, were satisfactorily eluted using a shorter column. Only one pair of maleimides appeared to be indistinguishable based on their retention times. These were the N-3-iodophenylmaleimide and N-4-iodophenylmaleimide. All other eluting maleimides could readily be distinguished by their retention times. Thus, the chromatographic stability and

differentiability of a substantial number of these maleimides has been demonstrated and could, in principle, permit labeling of a corresponding number of different antibodies to allow simultaneous determination of their homologous antigens.

Phase II, the collection of bloodstains:

A collection of aged bloodstains on various substrates was prepared. The bloodstains have been stored under different conditions to study the effect of different sample storage conditions on overall test results and to ultimately simulate dried bloodstains as they might be available from real casework.

Phase III, the preparation and purification of immunoglobulin fractions:

Several sources of ABO antisera were evaluated. The antiserum preparations were tested and fractionated to yield purified IgG and IgM fractions. The purification schemes which were developed for the required antibodies were used initially for production of small amounts of purified IgG and IgM fractions with specificities for A and B agglutinogens. These fractionation schemes are particularly well-suited to being scaled up to produce larger quantities of the antibodies. Another major advantage of these purification methods is their refinement to the point of being carried out simply. Scale-up of the purification sequence was confirmed by preparation of larger amounts of the immunoglobulin fractions.

Phase IV, the conjugation of a taggant to immunoglobulin:

For preliminary evaluation of the purified antibody fractions, conjugation with fluorescein isothiocyanate was carried out, with the conjugated antibodies then applied to the problem of typing dried bloodstains and other systems. The fluorescein isothiocyanate conjugation was performed on exceedingly pure IgG immunoglobulin fractions which were to be used as antibodies in subsequent tests. The fluorescein-labeled immunoglobulin was selected to provide a model of a tagged antibody derived from our fractionation sequence.

Phase V; testing of labeled antibodies and method evaluation:

Following conjugation of our antibodies, tests were carried out to check general activity and specificity. "Non-specific binding" of the tagged antibody would be an undesired result. Early tests with hair root sheath cells were excellent, but difficulty was experienced typing certain kinds of bloodstains as well as keratinized hairs. The latter had been crushed to provide better access for the antiserum to antigenic sites within the cortex.

Extensive tests were carried out to evaluate suitability of these labeled fractions for use with dried bloodstains. The fluorescent antibody conjugate was employed in attempts to type dried bloodstains by a direct immunofluorescence technique using epi-fluorescence microscopy. This dried bloodstain work was unsuccessful despite the fact that the reagent gave excellent results with fresh blood. These results parallel those of others who have attempted to apply immunofluorescence techniques to typing dried bloodstains. Thus it would appear that the previous workers' difficulties did not stem solely from impurities in their antiserum preparations.

Some of the difficulties encountered in our evaluation were attributable to autofluorescence of the blood as a result of changes which take place on drying. However, problems of this type should cause no difficulty with the proposed method which does not depend on fluorescence for detection of the antibody labels. Thus this autofluorescence problem is peculiar to the fluorescent antibody procedure, but other problems may

emerge as being of direct concern to this study. One such problem, e.g., indications of non-specific binding of the labeled antibody, will have to be solved for effective utilization of our technique.

Due to the unique simultaneous multi-antigen detection capabilities inherent in our proposed method, a novel means of dealing with non-specific binding suggests itself. One label could be used to tag an IgG which would not be expected to bind with the sample based on antibody-antigen specificities. After analysis the quantitative nature of our data would allow a correction factor for non-specific binding to be applied.

Our gc hardware-related work may be considered "Phase VI":

Very extensive work was required concerning design and fabrication of special new hardware for the planned pyrolysis - gc (pgc) work. Considerable modifications and modernizations of our basic gc system were undertaken to upgrade our instrument for "state-of-the-art" performance. During the course of the study, repeated repairs became necessary.

Three special ovens were designed and mounted for independent thermal control of a sample splitter (to permit use of capillary columns), a valve oven (for our ten-port valve) and a trap oven (for desorption of trapped pyrolyzate). Each of these ovens was separately controlled and regulated by a temperature control module we designed, built and calibrated using thermocouple sensors. Although splitter and valve ovens were to be maintained isothermally, some temperature programming facility was expected to be desirable for desorption from the trap. All of these assemblies entailed construction of mounts for attachment to or near the gc.

Interface plumbing was planned and constructed. Some modifications to the gc for capillary column use included incorporation of make-up gas inlet fittings and 1/16" hardware fittings for the detectors, etc. A sample splitter was mounted and tested for flow rate calibrations. This splitter was to be used for injected samples during early testing stages to establish, for example, gas chromatographibility and characteristic retention times of our synthesized maleimides. This would permit establishment of reproducible standard conditions as to: isothermal

column oven temperature or column oven program, injector and splitter temperatures and standard retention times of the actual molecules that would ultimately be liberated from tagged antibodies bound to a sample. Relative flame ionization detector (fid) sensitivities of the different maleimides and quantitative reproducibility were examined. Results indicated the desirability of using a shorter silica capillary column, 12 meters vs. 50 meters, for enhanced sensitivity and shorter retention times. These retention times would ultimately determine the identity of the specific maleimide released from a sample and would imply what antigen had originally been present in the sample. After preliminary work with glass capillary columns, we selected newly developed silica capillary columns because of their ruggedness and their convenience in use.

As part of the general refurbishment of our gc, testing and calibration was performed with 1/8" packed columns as well as glass and silica capillary columns. The latter were found most efficacious and were evaluated both in 50 and 12 meter lengths. The dual fid detectors and ecd were tested and calibrated after cleaning and overhauling. Wipe test evaluations of the cleaned ecd were performed to confirm operational safety; such tests were performed regularly at about three month intervals. Ecd checks were run in both DC and pulsed modes. Optimized pulsed ecd operation was planned for final work. The use of bromobenzene as an ecd response standard was considered.

Much of this gc work was plagued by repeated equipment breakdowns associated with substantial age of the basic instrument

and ancillary equipment. Often, parts were no longer available from the manufacturer. In particular, the basic pyrolyzer and associated pyrolyzer modifications consist of many delicate plumbing and electrical lines subject to breakage, aggravated by the high temperature at which they operate. A number of improvements, including fabrication of special Teflon parts, have somewhat improved the robustness and durability of the unit. Improved sampling techniques were elaborated, following studies of both open and closed end quartz capillary tubes. An adsorbent of activated charcoal fused with sodium silicate has been found stable and useful for sampling very low (sub-microliter) volumes of test solutions containing microgram quantities of solutes. Final flow rate adjustments for nitrogen or helium carrier gases, hydrogen and oxygen or air (for the fid) and 10% CH_4 in argon (for the ecd) were established. The use of a 12 meter rather than a 50 meter column has several justifications. Shorter retention time means faster elution and less chance of thermal degradation on-column as well as faster analyses. Also, faster eluting peaks will be less subject to peak broadening and will therefore emerge as sharper, higher peaks. Higher peaks mean an enhancement of signal-to-noise ratio and thus better (lower) detection limits for the maleimides. The shorter column was no worse than the longer column for the separation of the maleimides studied and would be best suited for the overall problem.

We had empirically observed very real limits imposed on our detection of maleimides during temperature-programmed gc operation because of septum bleed. Use of commercially available special "low bleed" septa was only marginally helpful. This problem was solved by the design, construction and installation of a novel combined needle guide and septum purge device. The device has allowed us to lower the effective detection limits by as much as a factor of 100. An article describing this has been accepted for publication in the Journal of Chromatographic Sciences.

A number of different techniques and methodologies have been explored with the configuration of the ten-port valve and associated pyrolyzer-trap-transfer lines. Some difficulties had initially been encountered. Impurities or decomposition products of materials used in the construction of the ten-port valve or trap evidently condensed on the gc column while it was cool and then eluted during temperature programmed runs. These peaks contributed excessive background and prevented the attainment of desired detection limits. As part of efforts to improve the quantitative desorption of pyrolyzate condensed in the trap and transfer of this material to the actual capillary column in a narrow "plug" for maximum peak sharpness, methods of both back-flushing and forward-flushing of the trap were explored, using varying trapping and desorption times and temperatures. We were eventually led to a specially designed, constructed and installed on-column cryogenic trapping device for cryofocusing samples directly onto our analytical column. A report of this device has been published in the Journal of High Resolution Chromatography and Chromatography Communications. This cryofocusing "tee" adapter was extensively tested and

found to work well. Its use results in compact sample plugs being condensed at the head of the silica capillary column so that narrow, well-resolved peaks are obtained during the subsequent temperature programming. This cryofocusing "tee" has been found useful in both of two different trapping/desorption modes. In one of these, the trap is placed in series with the column in both trapping and desorption operations. In the other,the pyrolysis/trap flow bypasses the column during the pyrolysis and trapping; during desorption, the flowpaths are switched by actuating the ten-port valve so that the trap is backflushed onto the column. The sample is then concentrated at the head of the column using the cryofocusing "tee" adapter, the temperature of which is held below ambient by using a controlled flow of liquid carbon dioxide. This flow is modulated in response to temperature changes in the column oven.

Despite "down time" problems of our ten year old gc continuing to plague us, with the manufacturer no longer making or supporting the instrument, and with service or replacement parts difficult to get or unavailable, our work has carried on. Very high turnover of technical staff resulted in considerable time lost for training. The burden of maintenance, trouble-shooting and repair has fallen on the principal investigators and the research staff, consuming much valuable time. Many positive results have nonetheless come out of this project.

Publications, Presentations and Theses Based on Project Work

R. Rothchild and P.R. De Forest, Journal of High Resolution Chromatography and Chromatography Communications, 5(6)321 (1982). "A Simple Device for On-Column Cryofocusing in Capillary Column Gas Chromatography".

P. R. De Forest and R. Rothchild, Journal of Chromatographic Science, in press. "Construction of Gas Chromatographic Syringe Needle Guide and Septum Purge Adapter".

P. R. De Forest, R. Rothchild, J. O'Connor, R. Fabrizio and F. Rios, "Simultaneous Determination of Several Antigens in Bloodstains," presented at the American Academy of Forensic Sciences, 33rd Annual Meeting, Los Angeles, CA, Feb. 18, 1981.

R. Rothchild, J. O'Connor, F. Rios, R. Fabrizio and P.R. De Forest, "Tagged Antibody Determination of Antigens Using Pyrolysis-Gas Chromatography," presented at the Mid-Atlantic Regional Meeting of the A.C.S., Wash., D.C. Jan. 9, 1981.

P. R. De Forest and R. Rothchild, "Approach to a Quantitative Gas Chromatographic Method for Simultaneous Determination of Several Antigens in Dried Bloodstains," presented at Metrochem '80, South Fallsburg, N.Y., Oct. 3, 1980.

P. R. De Forest and R. Rothchild, "Gas Chromatography in Immunology," presented at N.Y. Chromatography Society Minisymposium, NYC, June 11, 1980.

P. R. De Forest, R. Rothchild and R. Picciochi, "A Labeled Antibody Approach for Simultaneously Determining Several Antigens in Dried Bloodstains," presented at the Eastern Analytical Symposium, NYC, Nov. 2, 1979.

Richard Picciochi, "The Grouping of Dried Bloodstained Fabrics and Dried Blood Particulates by the Fluorescent Antibody Technique," M.S. thesis #594, Sept. 1980, John Jay College of Criminal Justice, City University of N.Y.

Ralph Fabrizio, "Design, Synthesis and IR, UV and MS Characterization of Diels-Alder Adducts of N-Substituted Maleimides and Analogs with Furfuryl Isothiocyanate," M.S. thesis #601, Feb. 1981, John Jay College of Criminal Justice, City University of N.Y.

END