PROGRESS REPORT P N-ABC-874
20 May 1989

US-ISRAEL CDR PROGRAM PROJECT NO. C5-187
GRANT NO. DPE-5544-G-SS-6020-00

Antibody Molecules as Templates and Catalysts for Organic Chemical Reactions

Principal Investigator:
Dr. Bernard S. Green
Department of Pharmaceutical Chemistry
The Hebrew University
P.O. Box 12065
91120 Jerusalem
ISRAEL
Tel: 972-2-428703. Home: 972-8-548455
FAX: 972-2-784010
Telex: 26132 NULMDIL (Attn: B. Green, Pharm. Chem.)

Co-principal Investigator:
Dr. Vicharn Vithayasai
Chairman, Department of Microbiology
Faculty of Medicine
Chiang Mai University
Chiang Mai 50 000
THAILAND
Tel. (053) 2211122, 221200 Ext. 1249
Telex: 43555 MEDCMU TH

Bernard S. Green

Summary. The field of antibody-induced chemical reactions has made enormous progress in the last two years and this area promises to have great impact, with applications in chemistry, biology, and medicine. In this research project we have (despite problems with personnel turnover, difficulties with some of the syntheses, and insufficiently close contact with our co-prinicipal investigator) made significant progress in better understanding the factors required in order to achieve decarboxylating catalytic antibodies. Our results suggest that: catalytic antibodies have little probablility of resulting from polyclonal antibody prepartations and that one must take the longer but more reliable route of obtaining monoclonal antibodies; the stability of the hapten and the hapten-protein carrier linkage is critical and must be sufficiently robust to withstand the array of

Rec'd in SCI JUN 13 1989

enzymes in biological fluids; the reactions chosen for study must have very low k_{uncat} ("background" or spontaneous reaction rates) in order to allow one to measure significant k_{cat}/k_{uncat} values. A system which meets these requirements, based on our experience, is now being actively studied. During the period of this grant a chapter has been published and a review article is in preparation both of which acknowledge AID-CDR support.

Introduction

In May, 1986, when Mr John R. Eriksson, wrote (to the Institute of Biological Research, my affiliation at that time) to inform us that our proposal had been funded, there had not yet appeared a single report of an antibody-catalyzed chemical reaction. The foresight and appreciation of the AID-CDR Program for truly novel research is, thus, particularly striking in the present example. Despite ideas which gave theoretical support for the proposed research, and our early feasibility studies, which underpinned the experimental approach (1), one reviewer commented: "This probably is not going to result in a new technology. It is unlikely to succeed at all!"

The AID-CDR Program is greatly appreciated for recognizing the validity of the other reviewer comments and accepting our rebuttal of the more pessimistic views. In the last two years, two other groups have published considerable results on antibody-mediated catalytic hydrolysis reactions (2,3). We have reported results demonstrating that antibody combining sites can act as templates to bring two molecules together in a desired pre-reaction configuration (4). The field of antibody-induced organic chemical reactions is now attracting considerable attention (5) and promises to have enormous impact on chemistry, biology and medicine in the coming years.

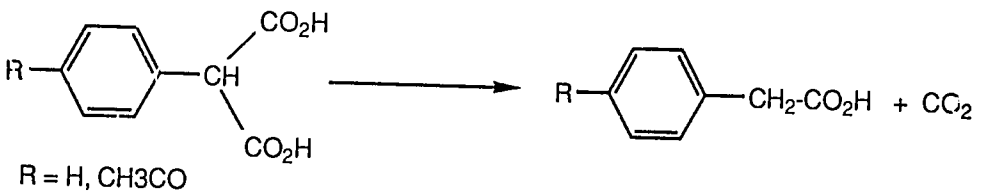
Clearly, the field of catalytic antibodies has "arrived". However, in the leap from being considered "unlikely to succeed" to acceptance as an approach for "open[ing] up [a] new strategy for protein engineering" (5d) many important questions have been asked but very few have been answered. Perhaps the major one relates to the generality of this technique: What is the scope of antibody-catalyzed reactions? In a critical review article (6), (which kindly acknowledges AID-CDR support) these questions are discussed and the biotechnological implications of this field are summarized.

The present research is thus particularly important in this context: it may considerably expand the scope of antibody-catalyzed reactions. Decarboxylation involves carbon-carbon bond breaking, a new class of antibody-catalyzed reactions; in addition, a whole new family of catalytic antibody-producing haptens may be identified.

Research Report

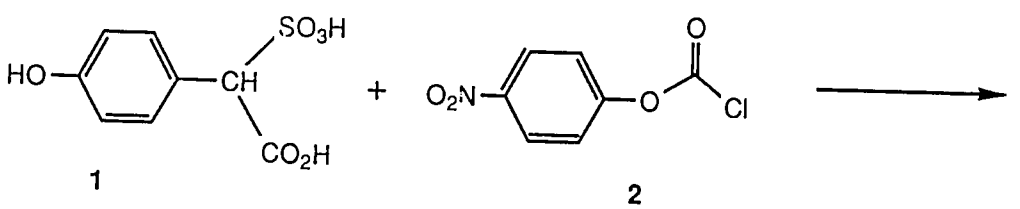
A. Hapten Synthesis

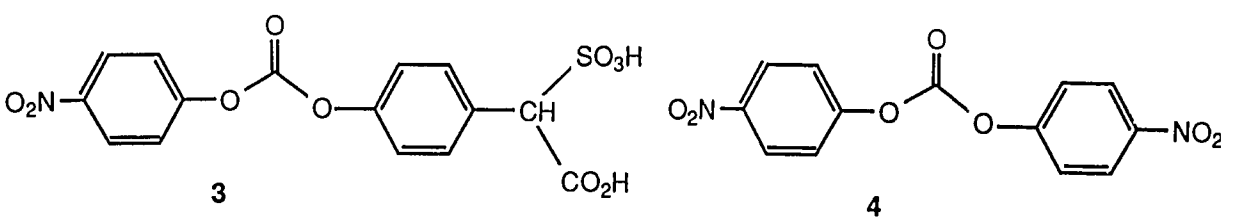
As the synthesis of some of the more interesting hapten compounds proved to be quite time-consuming, we focused primarily on the commercially available hapten**1**, a sulfoacetic acid derivative. Anti-**1** antibodies would be expected to catalyze the decarboxylation of phenylmalonic acid (Eq. 1) or a para-substituted derivative. This material is supplied as the Na salt; in order to carry out further chemical reactions and use**1** as a hapten we needed the free sulfonic acid. This was prepared by using a Dowex (acid form) column: the product was lypholyzed and found to be pure (NMR and C,H,S microanalysis). In order to link **1** to a protein carrier several methods were tried.



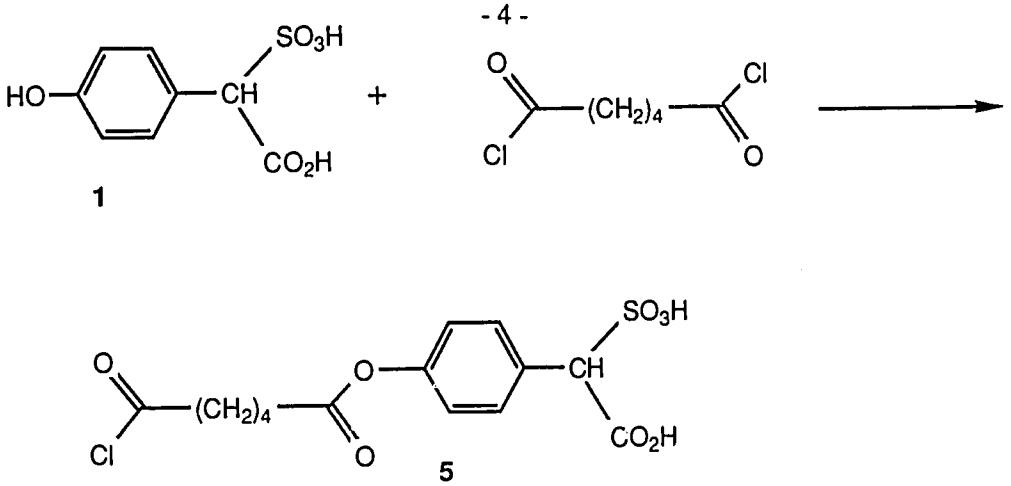
Eq. 1

1. p-Nitrophenylchloroformate (**2**) to give a carbonate (**3**). The idea here was that the p-nitrophenyl group of **3** could be readily displaced by an amino group of the protein carrier to give a carbamate linkage. The desired product **3** was not formed under a variety of reaction condiditions; one of the products formed was bis-p-nitrophenyl carbonate (**4**) apparently formed via a disproportionation reaction.

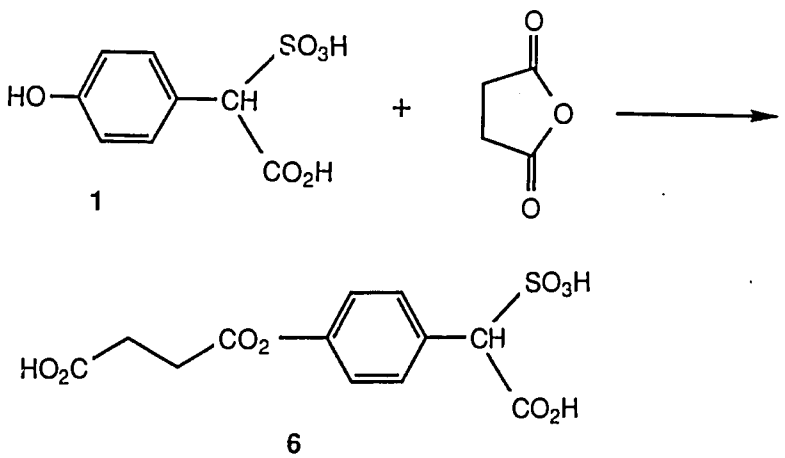




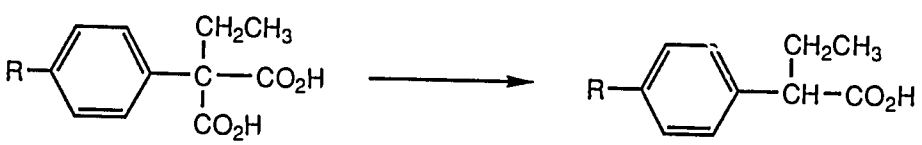
2. Adipoyl chloride method. When **1** was treated with adipoyl chloride in DMF and warmed, reaction took place and **1** was no longer present. The product **5** was directly used to form protein conjugates.



3. Succinic anhydride method. Treatment of **1** with succinic anhydride gave the desired product **6** which was characterized and used to prepare protein conjugates.



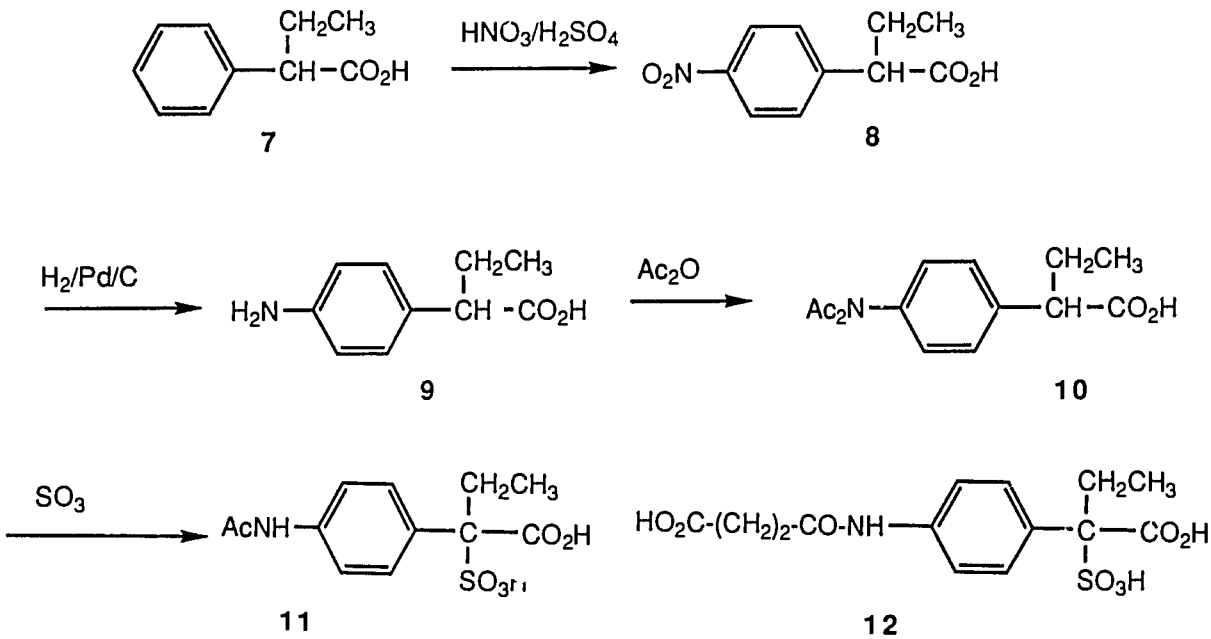
B. New Hapten Synthesis. Because of the ester-stability problem (discussed below) an amide instead of an ester linkage was desired between the hapten and protein carrier. In addition, we chose a substrate which would give a decarboxylated product maintaining an element of chirality and therefore set compound **11** as the target for synthesis. Anti-**11** antibodies would be expected to catalyze the decarboxylation of phenyl ethyl malonic acid, or para-dereivatives of this substance (Eq. 2). The 3-phenylbutyric acid has a chiral center.



Eq. 2

R = H, CH_3CONH

The commercially available starting material **7** was readily nitrated to give the p-nitroderivative **8**, which was catalytically hydrogenated to **9** and then acetylated to give **10**. (All compounds **8-10** were characterized by NMR and microanalysis.) Sulfonation of **10** to the desired **11** using pyridine.SO3 complex did not succeed and this step is currently under study. When this is achieved, the actual hapten will be the analog **12** which contains a carboxylic acid for coupling to a protein.



C. Preparation of conjugates

1. Adipoyl chloride product. The product **5** was directly added to a solution of BSA in PBS buffer (pH 7.9, adjusted by adding 1M NaOH) and stirred for 1hr at RT, and then overnight in cold room. After dialysis against 0.01M NaHCO3 and water in cold room, the mixture was separted into an insoluble fraction and a soluble fraction, both of which were lypholyzed, characterized, and used to raise antibodies. Infrared spectra of the conjugates showed the presence of the hapten moiety. The number of haptens bound per BSA was estimated using the trinitrobenzenesuifonic acid method (7); the insoluble fraction contained 3-4 haptens/BSA while the soluble fraction contained 7-9 haptens/BSA.

2. Succinic anhydride product. The product **6** was dissolved in pyridine, treated with a water-soluble carbodidmiide reagent (1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-p-toluenesulfonate) and then added to a solution of BSA in PBS buffer and stirred overnight. The solution was dialyzed as above; the insoluble fraction was minor and discarded. Lypholization yielded conjugate containing the hapten (IR spectrum) and

Lypholization yielded conjugate containing the hapten (IR spectrum) and having 5-6 haptens/BSA.

3. KLH conjugates. Conjugates of **5** and **6** with KLH were prepared and characterized in the same way.

Lability of ester conjugates. Although the conjugates prepared from **5** and **6** appeared to be stable (e.g., extensive dialysis did not cause any significant breakdown) and we were apparently getting a good antiserum response, we began to suspect problems when there was no inhibition of binding of antisera to antigen when even high levels of free hapten were added to the test solutions. Further experiments with serum and with enzymes, such as hog liver esterase (HLE), which may be expected to be present in serum, showed that the antigens were being partially destroyed by HLE and by components present in serum. These results were obtained following the initial results with antibody-promoted decarboxylation (which were therefore now explained as being due to absence of sufficient specific antibody). We are awaiting at present the antibody preparations raised against **12**; the antigen will be carefully monitored for stability in serum.

Immunization.

A. Polyclonal Antibodies. New Zealand white rabbits were used for preparation of polyclonal antibodies. The antigens (2 mg) were emulsified in a complete Freund adjuvant, 1:1, and injected intradermally in 10 sites; four booster injections were given at 2-4 week intervals. Immune sera were collected 10 days after the last injection. By precipitation (8), using hapten coupled to a different protein, there appeared to be a reasonable level of anti-hapten antibodies present, but when, later, inhibition of antiserum binding with different levels of the free hapten was attempted, there was no appreciable inhibition of binding.

B. Monoclonal Antibodies. Initial experiments were perfomed to raise monoclonal antibodies but when the conjugates were discovered to be labile, these were not further studied. The experience will allow more rapid and efficient monoclonal antibody production with the antibodies to be raised against **12**.

Decarboxylation studies.

Phenyl malonic acid was studied according to Eq.1. The decarboxylation in aqueous solution followed the kinetics reported n the literature (9). Initial studies showed that polyclonal antibody preparations induced decarboxylation at 40°. However controls, using normal rabbit antibody

preparations also showed, albeit at a lower rate, induced decarboxylation. In order to see significant differences between antibody-catalyzed decarboxylation and nonspecific decarboxylation it was concluded that it is necessary to obtain pure, monoclonal antibody, specific for the hapten. This is now being pursued.

Analytical System.

To determine the amount of reaction, a variety of methods have been used. Of the analytical techniques which we have set up to use for accurate kinetics, gas chromatography using a thermal conductivity detector and a Chromosorb 102 column (Supelco) is most accurate and reliable. The solutions must be acidified to eliminate HCO_3^- ion if the pH is basic. A sensitive visual test wwhich we also use is to prepare a solution of bromthymol blue (0.04% aqueous solution) and bubble the exit gases of the reaction into this solution. As CO_2 enters the solution and is converted to H_2CO_3, the bromthymol solution changes from blue to yellow. The quantity of CO_2 can also be measured accurately and continuously by manometer (10). For a simple, initial method of detection of reaction, our systems were particularly amenable to TLC since reactant and product have significantly different Rf values (eluant was 1% acetic acid -- which eliminates tailing of carboxylic acid spots -- in ethyl acetate containing varying amounts of methanol).

Collaboration

Although there have been some communication between the Israeli and Thai researchers, this has been far from adequate. The Israeli PI should probably have visited Thailand at an early stage of the research, during the first year, (we did meet before the submitting of the original proposal) and ironed out more explicitly exactly what we would jointly do. The Israeli PI is planning to visit Thailand in the near future and it is hoped to clarify and better coordinate the work that is being done in both labs. It is hoped that a Thai coworker will come to work in Israel as well.

The importance of biotechnology for the future of countries other than the Western and highly industrialized ones is being increasingly recognized (see, for example, the review of the book "The Biotechnological Challenge", by S. Jacobsson, A. Jamison, and H. Rothman, Cambridge University Press, 1986, in Chemical & Engineering News, March 23, 1987 issue, pp. 77-78: ' Biotechnology for the Third World", by R. P. Morgan). The antibody approach should certainly be included as one attractive way to achieve solutions to a variety of problems. This recognition of the importance and significance of the results which may emerge from such research should encourage and promote fruitful collaboration.

Personnel

We have had some unsatisfactory experiences with personnel on this project. The postdoctoral fellow who began the experimental work on this project (Dr. Sara Stelzer) terminated her involvement after some 8 months. She had just managed to get involved in the synthesis and the idiosyncracies of the compounds (e.g., hapten **1**) when she decide to leave. The carry-over to the student who took over from her was not sufficient. This student (Mr. Najim Lutfy) worked for a year and found the project too demanding. He repeated much of Dr. Stelzer's work and extended this somewhat. An excellent new student with a backgound in both chemistry and biology (Mr. Dan Tawfik) has recently joined the project and, it is hoped, his involvement will greatly help the successful achievement of results.

Publications.

Two publications have been prepared during the course of this reserach which deal with catalytic antibodies. The first, a chapter in the series Advances in Biotechnological Processes, has appeared this year. Two reprints are being submitted separately. The second, a review to appear in Trends in Biotechnology, will be published shortly; the title will be "Catalytic Antibodies". Both of these publications acknowledge the support of the AID-CDR.

Bernard S. Green. "Monoclonal antibodies as catalysts and templates for organic chemical reactions", in Mizrachi A (ed): "Monoclonal Antibodies: Production and Application", Advances in Biotechnological Processes, Vol 11, 359-393, 1989.

Bernard S. Green and Dan Tawfik, "Catalytic Antibodies", Trends in Biotechnology, Elsevier Publications, Cambridge, J. Hodgson, ed., to be published.

References

1. Kohen F, Kim JB, Lindner HR, Eshhar Z, Green BS: Monoclonal immunoglobin G augments hydrolysis of an ester of the homologous hapten: An esterase-like activity of the antibody containing site? FEBS Lett., 111:427, 1980.

2. K.D.Janda, S.J.Benkovic and R.A. Lerner. Catalytic antibodies with lipase activity and R or S substrate selectivity. Science, 244, 437-440, 1989; and other references therein.

3. K.M.Shokat, CJ.Leumann, R.Sugasawara, and P.G. Schultz. A new strategy for the generation of catalytic antibodies. Nature, 338,269-271, 1989; and other references therein.

4. Balan A, Doctor BP, Green BS, Torten M, Ziffer H: Antibody combining sites as templates for promoting selective organic chemical reactions. JCS Chem Comm 106-108, 1988.

5. (a) J.L. Marx, Science, 234, 1497 (1986); (b) D.E. Hansen, Nature, 325, 304 (1987); (c) R.J. Massey, Nature, 328, 457 (1987); (d) R.M. Baum, Chemical & Engineering News, 6 April 1987 issue, pp. 30-33. For recent reviews see: Schultz PG: The interplay between chemistry and biology in the design of enzymatic catalysts. Science 240:426-433, 1988; Lerner RA, Benkovic SJ: Principles of antibody catalysis. BioEssays 9, 107-112, 1988; and reference 6.

6. Green BS: "Monoclonal antibodies as catalysts and templates for organic chemical reactions", in Mizrachi A (ed): "Monoclonal Antibodies: Producvtion and Application", Advances in Biotechnological Processes, Vol 11, 359-393, 1989.

7. A.F.S.A.Habeeb, Anal. Biochem. **14**, 328-336 (1966). See also: Anal. Biochem. **64**, 284-288 (1975).

8. M. Sela, S.Fuchs, and R. Arnon, Biochem. J., 85, 223-235 (1962).

9. G.A. Hall and E.S. Hanrahan, J. Phys. Chem., **69**, 2402-2406 (1963).

10. See, for example: J.W. Thanassi and J.S. Fruton, Biochemistry, **1**, 975 (1962).