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Reversible Acylation and Inhibition of Aggregation of Platelets by Substituted Maleic Anhydrides

JOSEPH A. WALDER, BRADLEY T. HYMAN, MICHAEL J. HELLER, ROXANNE Y. SHISHIDO, AND IRVING M. KLOTZ

Department of Biochemistry and Molecular Biology and Department of Chemistry, Northwestern University, Evanston, Illinois 60201

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SUMMARY

WALDER, JOSEPH A., HYMAN, BRADLEY T., HELLER, MICHAEL J., SHISHIDO, ROXANNE Y. & KLOTZ, IRVING M. (1977) Reversible acylation and inhibition of aggregation of platelets by substituted maleic anhydrides. *Mol. Pharmacol.*, 13, 407-414.

The antiprostaglandin action of aspirin due to the acetylation of the enzyme prostaglandin synthetase is manifested by inhibition of the so-called release reaction and consequent abolition of secondary-phase aggregation. The maleic anhydrides, a series of reversible acylating agents, have now been shown to produce the aspirin-type defect in platelet aggregation. The antiplatelet effect of aspirin is irreversible. With 2,3-dimethylmaleic anhydride, however, the antiplatelet effect disappeared in approximately 80 min at 25°. Hence the persistence of the lesion when caused by aspirin results from the stability of the acetyl linkage formed in its reaction with prostaglandin synthetase. This suggests that the modification occurs at either a lysine or an NH_2-terminal amino group. That such is the case was confirmed by the observation that 3,4,5,6-tetrahydrophthalic anhydride and 2,3-dimethylmaleic anhydride, both amino-specific acylating agents, were able to effect the aspirin-type lesion. Thus, upon deacylation, prostaglandin synthetase regains its activity and platelet function returns to normal. Accordingly, it may be possible to develop a clinically acceptable, reversible acylating agent with short-acting, aspirin-like effects.

INTRODUCTION

That aspirin is an effective acylating agent for proteins (1, 2) and a potent irreversible inhibitor of prostaglandin biosynthesis (3, 4) is well established. Only recently has it been shown that these activities are causally related. The block in prostaglandin production results from the irreversible inactivation of prostaglandin synthetase, the microsomal enzyme responsible for the conversion of arachidonic acid to the labile cyclic endoperoxide intermediates of prostaglandin biosynthesis (5-7). Majerus and co-workers (8, 9) demonstrated that aspirin acetylates this enzyme and that the rate of acetylation correlates well with the onset of the antiprostaglandin effects of the drug. The acetylation of prostaglandin synthetase was relatively specific, occurring at concentrations 10-100 times lower than necessary to achieve modification of human serum albumin and hemoglobin. In addition, the reaction was competitively inhibited by the substrate for the enzyme, arachidonic acid. Taken together, these observations indicate that aspirin inhibits prostaglandin synthetase by acetylating a critical residue at its active site. The irreversible nature of the lesion suggests that acetylation occurs

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either at the ϵ-amino group of a lysine residue, as in the reaction of aspirin with human serum albumin and hemoglobin, or at the NH_2-terminal amino group of the enzyme, in either case leading to the formation of a stable amide linkage.

These observations raise the possibility that deacylation of prostaglandin synthetase would restore its activity and thereby reverse the antiprostaglandin effect. If this were true, it would be possible to develop a reversible acylating agent with short-acting, aspirin-like effects. Such an agent would greatly extend the experimental utility of acylating reagents as antiprostaglandins, and would be clinically superior to aspirin itself. The latter follows from the finding that a number of patients with subclinical or overt hemostatic abnormalities develop severe bleeding disorders as a result of the ingestion of aspirin (10). This is due in large measure to the antiplatelet effect of the drug, caused by the acetylation of platelet prostaglandin synthetase. The platelet defect is irreversible; that is, once acetylated, a platelet remains afflicted throughout the remainder of its lifespan. Platelet function returns to normal and the bleeding diathesis resolves only after a sufficient number of acetylated platelets are turned over and replaced by unaffected ones. This generally requires 4–7 days, a time often too long to wait for spontaneous remission of the bleeding disorder. To terminate the bleeding diathesis iatrogenically, transfusions must be given, a treatment not without its own risks. Thus the patient who presents a severe bleeding disorder following the ingestion of aspirin poses a difficult clinical situation. Use of a reversible, aspirin-like acylating agent would substantially mitigate this problem.[1]

[1] Many other pharmacological and toxicological actions of asprin appear to be due, at least in part, to prostaglandin synthetase blockade in other tissues (11). Since the inhibition of the enzyme at these sites must also be irreversible, the related effects suggest further advantages of a reversible acylating agent over aspirin. Reversible inhibitors of prostaglandin synthetase which inhibit the enzyme competitively are known. The duration of action of such compounds depends on the rate at which they are eliminated or inactivated by metabolic conversion. A reversible acylating agent offers the potential advantage over these compounds of little patient variability in duration of action, as the mechanism for terminating the effect of the drug is built into the drug itself.

Protein modification with reversible acylating agents has been studied extensively (12). The substituted maleic anhydrides have been examined in particular detail and offer a series of acylating agents which form amide linkages of varying lability. Therefore, in an effort to demonstrate the potential reversibility of the acylation-induced prostaglandin synthetase lesion and ultimately to develop a reversible acylating agent with aspirin-like properties, we have examined the effects of these anhydrides on platelet aggregation. In addition, since the specificity of these agents is known, they provide a probe into the nature of the active site of prostaglandin synthetase.

MATERIALS AND METHODS

Aspirin was obtained from J. T. Baker. Maleic acid, maleic anhydride, 3,4,5,6-tetrahydrophthalic anhydride, and 2,3-dimethylmaleic anhydride were purchased from Aldrich. 3.4,5,6-Tetrahydrophthalic acid was prepared by hydrolysis of the corresponding anhydride. All organic solvents were obtained from commercial sources and were of the highest purity available.

ADP and epinephrine, used to induce aggregation, were products of Sigma Chemical Company and Parke, Davis and Company, respectively. The stock ADP was 0.25 mM in 0.15 M NaCl solution. Epinephrine was obtained as a standard 1:1000 solution in 0.15 M NaCl and was diluted to a concentration of 0.8 mM with 0.15 M NaCl solution. Stock solutions of ADP and epinephrine were stored frozen in 1-ml aliquots at −20° and thawed immediately before use.

Preparation of platelet-rich plasma. Whole blood was drawn by venipucture from healthy adults, both male and female. The donors had not taken any medication for at least 14 days and had not eaten for at least 3 hr prior to venipuncture. One part of anticoagulant 3.2% triso-

dium citrate, pH 7.0, was added to 9 parts of whole blood.

Citrated whole blood was centrifuged at 82 × *g* for 10–15 min. The supernatant, platelet-rich plasma, was then separated from the red cells. The blood was next centrifuged at 844 × *g* for 15 min to yield platelet-poor plasma. If the platelet-poor plasma was turbid, the platelet sample was discarded.

The platelet count of the platelet-rich plasma was determined with a hemocytometer, on an aliquot diluted 100-fold with a 1% solution of ammonium oxalate. For aggregation studies, the platelet-rich plasma was adjusted to a final concentration of approximately 400,000 platelets/mm^3 with platelet-poor plasma. Platelet-rich plasma was incubated in a 25° water bath until used.

Platelet aggregation studies. Platelet aggregation was followed by the turbidimetric method of Born (13) on a Cary 14 spectrophotometer. An apparatus was designed which provided constant temperature (37°) and continuous stirring (1600 rpm) during the experiment.[2] The experimental procedure was as follows: 0.75 ml of platelet-rich plasma was transferred into a cylindrical cuvette (Chronolog) and incubated at 37° for 5 min in the cell compartment; stirring was initiated, and either epinephrine or ADP was added to induce aggregation.

Concentrated stock solutions of compounds to be tested were prepared in methanol immediately before use. An organic solvent was necessary for this purpose, since many of the compounds were not sufficiently water-soluble and were susceptible to hydrolysis. A number of organic solvents at concentrations up to 5% by volume were screened for compatibility with platelet aggregation. Of the solvents examined, methanol had the least effect on aggregation and produced no detectable influence at concentrations up to 1.5%. In actual tests the final concentration of methanol was usually 0.5%. Immediately after introduction of the appropriate volume of stock solution into a sample of platelet-rich plasma, an aliquot was taken for aggregation, and the remainder was incubated in a 25° water bath. Aliquots were taken for aggregation every 20–30 min thereafter. Control platelets were incubated under the same conditions and were tested intermittently to ascertain the normal aggregation pattern. Platelets generally retained viability for 2–3 hr. All studies were concluded with a control run to ensure that the platelets had retained viability throughout the entire experiment.

To test 2,3-dimethylmaleic acid, the anhydride was hydrolyzed immediately before use and administered as a 50% methanol–water solution.

[2] A description of this apparatus will be published elsewhere.

RESULTS

In the turbidimetric method, the decrease in absorbance of platelet-rich plasma, or increase in transmittance, accompanying platelet aggregation is followed (13). Figure 1A shows the typical epinephrine-initiated biphasic aggregation pattern. During the initial phase, platelets retain their morphological integrity. The second phase of aggregation is initiated by the so-called release reaction, in which the contents of the dense granules within the platelets are extruded. Of these contents, ADP appears to be the agent directly responsible for induction of the secondary phase (14). In this process platelets become irreversibly enmeshed in the platelet plug.

The release reaction is dependent on the activity of prostaglandin synthetase (7) and therefore is blocked by aspirin. This is evidenced by the abolition of the secondary phase of epinephrine-induced aggregation, with little or no effect on the primary phase (Fig. 1B) (15). The lesion is complete immediately (operationally this is defined throughout as less than or equal to 5 min, the time allowed for the sample to equilibrate to 37°) after introduction of aspirin at a concentration of only 25–50 μM. Other aggregating agents can be used to demonstrate the aspirin lesion, but none so unequivocally as epinephrine. The pattern of epinephrine-initiated aggregation, as well as the aspirin defect, was found to be independent of epinephrine concentration from

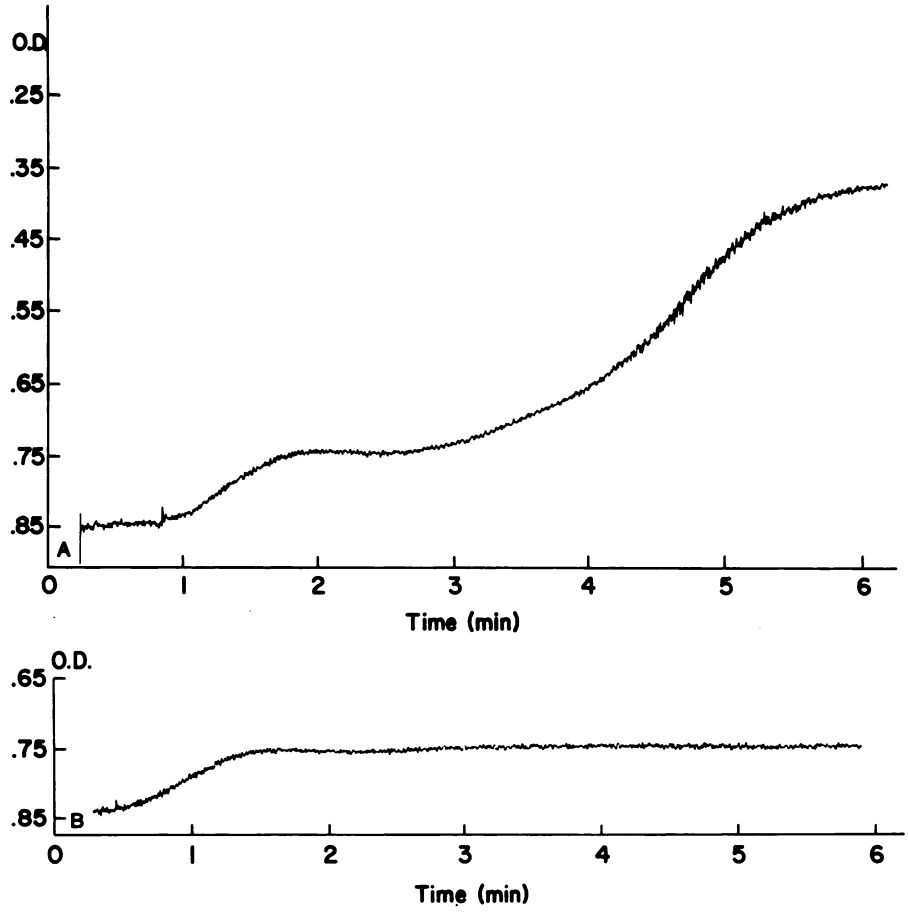


FIGURE 1

A. Typical epinephrine-initiated biphasic aggregation pattern. The epinephrine concentration was 40 μM. B. Abolition of secondary-phase aggregation in platelets treated with 50 μM aspirin.

10 to 80 μM. Therefore a standard concentration of epinephrine of 40 μM was used uniformly to initiate aggregation.

Acetone, acetonitrile, dioxane, isopropyl alcohol, ethanol, and methanol were screened for compatibility with platelet aggregation. Of these solvents, methanol was the most compatible with aggregation and produced no detectable effect at concentrations up to 1.5% by volume. Furthermore, aspirin delivered from methanol stock solution gave the same defect as when administered in 0.15 M NaCl solution. Consequently methanol was used to prepare stock solutions of all agents to be tested.

Maleic anhydride, 3,4,5,6-tetrahydrophthalic anhydride, and 2,3-dimethylmaleic anhydride each abolished the secondary phase of epinephrine-initiated aggregation, with little or no effect on the primary phase (Fig. 2A). The exact concentration necessary to effect this lesion varied with platelets obtained from different individuals, from approximately 2 to 4 mM for each of the anhydrides. Generally maleic anhydride was slightly more potent than the other two. As with aspirin, the defect was complete immediately after introduction of the agent. At concentrations of anhydride greater than required to abolish the secondary phase, a progressive decrease in the amplitude of the primary phase resulted as well. In some instances, even though the lesion appeared complete, a secondary decrease in absorbance of very

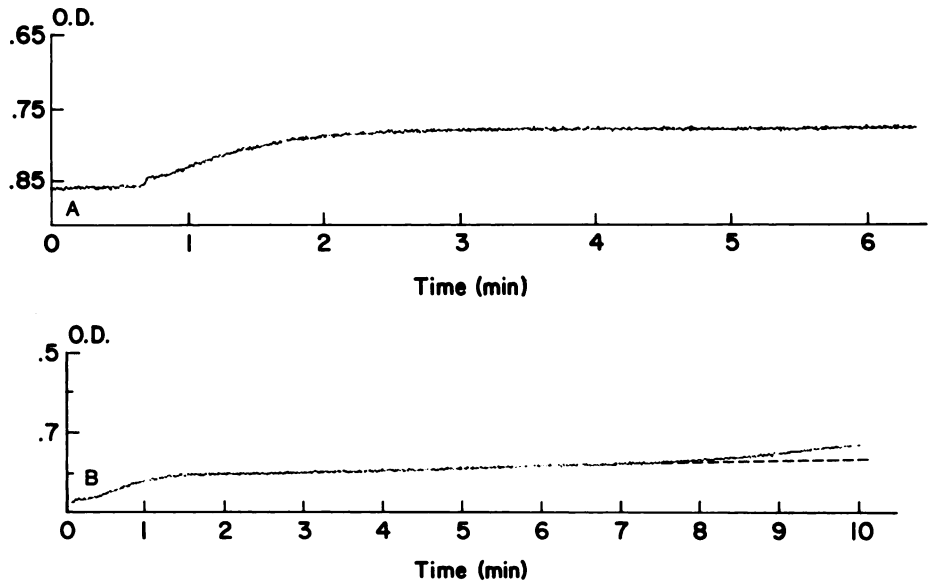


FIGURE 2

A. Abolition of secondary-phase aggregation in platelets treated with 2.5 mM maleic anhydride. B. Incomplete lesion of secondary-phase aggregation. The platelets were treated with 3.75 mM 3,4,5,6-tetrahydrophthalic anhydride. Extension of the linear portion beyond 8 min is indicated by the dashed line.

shallow slope began 8–15 min after the initiation of aggregation (Fig. 2B).

The defect in the secondary phase of aggregation caused by aspirin, maleic anhydride, or 3,4,5,6-tetrahydrophthalic anhydride remained unchanged throughout the 2–3 hr during which the control platelets retained viability. However, the inhibition of secondary-phase aggregation due to 2,3-dimethylmaleic anhydride was reversed in approximately 80 min (Fig. 3). Recovery from the antiplatelet effect of this agent appeared with every platelet sample tested. In total this represented about 10 studies on samples taken from six individuals.

Hydrolysis of the anhydrides to the corresponding diacids generally produced a drop in the pH of platelet-rich plasma of 0.2–0.3 unit. To assess the effects of the pH drop and any potential activity of the diacids themselves, the diacids as such were administered. They showed little or no effect on aggregation at concentrations at which the anhydrides produced the complete lesion. An equivalent amount of HCl also had no significant effect on aggregation.

ADP itself can initiate aggregation and, at sufficiently high concentrations, does not require the release of endogenous ADP for initiation of the secondary phase. Hence at high concentrations of ADP (25 μM) aggregation was monophasic and unaffected by aspirin (16). Furthermore, aspirin-treated platelets that completed the primary phase of epinephrine-initiated aggregation underwent secondary-phase aggregation in response to exogenous ADP. Platelets acylated with the maleic anhydrides responded to exogenous ADP exactly as did aspirin-treated platelets (Fig. 4).

DISCUSSION

The antiplatelet effect of aspirin caused by the acetylation of prostaglandin synthetase is irreversible. The persistence of the lesion could be accounted for by either (a) the stability of the acetyl linkage formed in the reaction of aspirin with prostaglandin synthetase, (b) irreversible changes in the structure of prostaglandin synthetase secondary to acetylation, or (c) permanent defects in related functions due to the antiprostaglandin effect. To distinguish

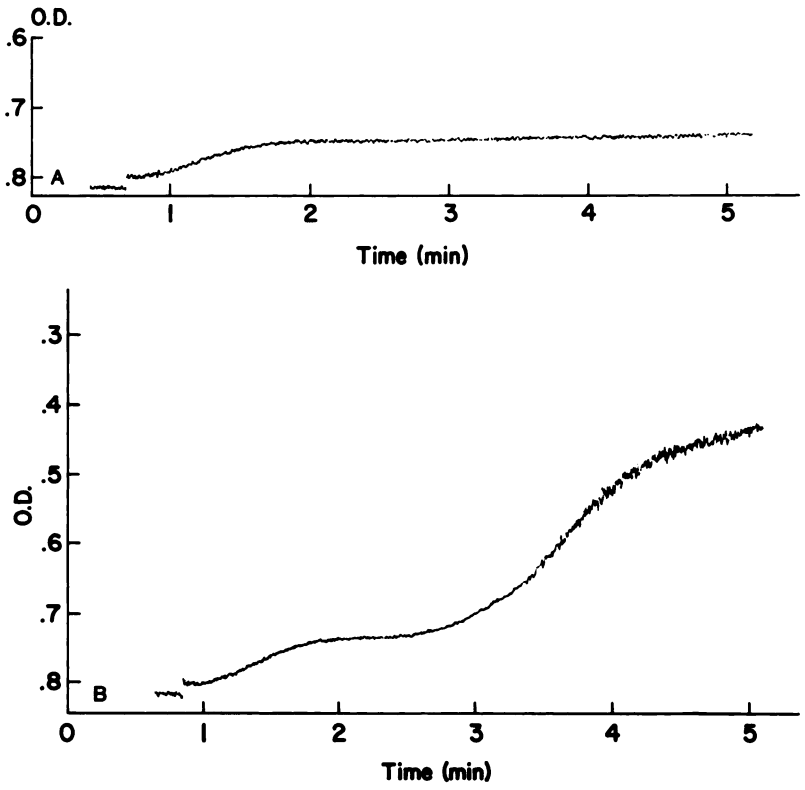


FIGURE 3

A. Abolition of secondary-phase aggregation by 2.5 mM 2,3-dimethylmaleic anhydride. B. Recovery of secondary-phase aggregation 80 min after introduction of 2,3-dimethylmaleic anhydride (experiment of Fig. 3A).

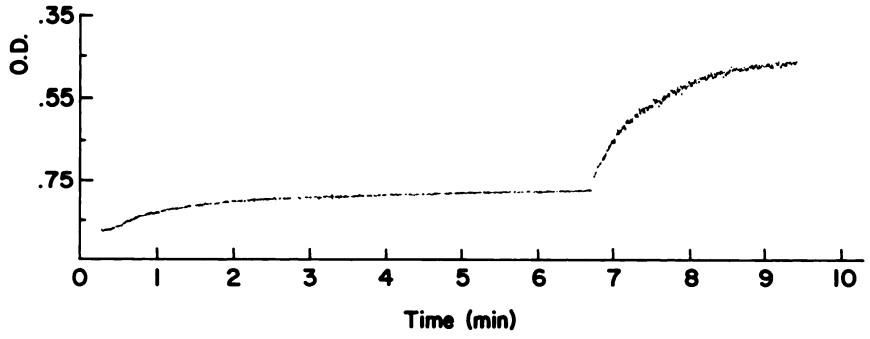


FIGURE 4

Secondary-phase aggregation in response to exogenous ADP in platelets treated with 2.5 mM maleic anhydride. The primary phase of aggregation was initiated with 40 μM epinephrine. ADP was added at 7 min.

among these possibilities, we studied the effects of a series of reversible acylating agents, the substituted maleic anhydrides, on platelet aggregation.

Each of the substituted maleic anhydrides produced an aspirin-like defect in epinephrine-initiated platelet aggregation (Fig. 2A). That this was due to blockade of the release reaction was established by the demonstration that the anhydride-treated platelets undergo secondary-phase aggregation in response to exogenous ADP (Fig.

4). Thus, if the release reaction, and hence the liberation of endogenous ADP, had occurred, secondary-phase aggregation would have resulted. In addition, the responses elicited by ADP eliminated the possibility of any generalized defect in secondary-phase aggregation.

The reversal of the aggregation defect only in those platelets treated with 2,3-dimethylmaleic anhydride (Fig. 3) implicated acylation as the cause of the lesion. Of the maleic anhydrides examined, this derivative forms the most labile acyl adducts (see below). No other distinguishing properties of this agent are evident that could explain the recovery from its antiplatelet effect. Because the anhydrides are rapidly hydrolyzed, an alternative source of their antiplatelet action might be the diacids formed. This possibility was ruled out by the demonstration that the diacids have little or no effect on aggregation at concentrations at which the anhydrides produce the complete lesion.

Although aspirin acetylates several platelet constituents, only the modification of prostaglandin synthetase correlates with its antiplatelet effect (9). Like aspirin, the maleic anhydrides undoubtedly acylate a number of platelet proteins. Since the maleic anhydrides produce the same lesion as does aspirin and have the same molecular mode of action (acylation) and onset of effect as aspirin, we conclude that the critical site of modification is the same as for aspirin.

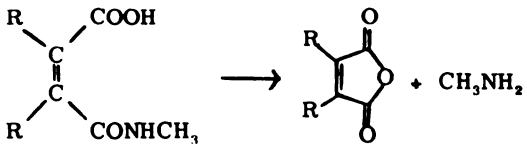
The spontaneous recovery of platelets treated with 2,3-dimethylmaleic anhydride demonstrates that upon deacylation prostaglandin synthetase regains its activity and that there are no permanent defects in platelet function secondary to the antiprostaglandin effect of prostaglandin synthetase inhibition. Hence the persistence of the lesion when caused by aspirin presumably results from the stability of the acetyl linkage formed in its reaction with prostaglandin synthetase. This suggests that the modification occurs at either a lysine or an NH_2-terminal amino group. The observation that 3,4,5,6-tetrahydrophthalic anhydride and 2,3-dimethylmaleic anhydride are able to effect the aspirin lesion confirms this conclusion, since these agents acylate amino groups specifically (17, 18); acyl adducts that might be formed with side chains of other amino acids are far too labile to exist for any length of time.

The mechanism for the hydrolysis of substituted maleamic acids has been examined in detail (19). The intramolecular carboxyl group, in the protonated state, attacks the amide function to release the amine and form the intermediate anhydride. Subsequently the anhydride is hydrolyzed to give the diacid. The rates for release of methylamine from several substituted *N*-methylmaleamic acids are shown in Table 1 (19). If the maleic acid derivative is assigned a rate of 1, the 3,4,5,6-tetrahydrophthalic and the 2,3-dimethylmaleic derivatives have relative rates of 800 and 16,000, respectively. From these relative rates one can estimate that, since the resolution of the defect due to 2,3-dimethylmaleic anhydride required approximately 80 min at 25°, the reversal of the antiplatelet effects of 3,4,5,6-tetrahydrophthalic anhydride and maleic anhydride should occur in about 27 and 21,300 hr, respectively. Hence platelets treated with these agents would not be expected to regain activity in the 2–3 hr during which they could be studied. This agrees with our experimental observations.

Complete recovery from the lesion caused by 2,3-dimethylmaleic anhydride is demonstrated in Fig. 3. At 25° approximately 80 min were required to reach the

TABLE 1

Rates of hydrolysis of substituted N-methylmaleamic acids at 39° and ionic strength 1.0 (19)



Compound	k_{obs}
	min^{-1}
R = H	3.91×10^{-3}
R + R = $-(CH_2)_4-$	3.13
R = CH_3	62.6

degree of deacylation necessary to restore normal platelet function. Aggregation initiated at intermediate times manifested a secondary wave of nearly normal morphology following a lag period after the primary wave. With time, the duration of the lag phase decreased until the pattern of aggregation finally returned to normal. This sequence obtained because at the temperature at which aggregation was performed, 37°, deacylation was accelerated, probably about 3–4-fold over the rate at 25°, and progressed sufficiently during the experiment itself to reverse the antiplatelet effect. In some instances aggregation studied immediately after introduction of 2,3-dimethylmaleic anhydride evidenced a secondary phase after a lag period of about 10–15 min. That this represented restoration of function rather than simply an incomplete lesion (as in Fig. 2B) was clear from the normal morphology of the secondary wave.

Although the maleic anhydrides proved useful for demonstration of the potential reversibility of the acylation-induced prostaglandin synthetase lesion, these agents are, of course, far too labile for any clinical use as well as for many experimental applications. Nevertheless, their behavior suggests that it would be worthwhile to prepare derivatives of the maleic acids and of other carboxylic acids with intramolecular catalytic moieties in a search for a clinically acceptable, short-acting acylating agent with aspirin-like effects.

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