The Esterase-Like Activity of Serum Albumin May Be Due to Cholinesterase Contamination

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Purpose. The "esterase-like activity" of human serum albumin (HSA) is described in the literature, but a contamination of commercially available HSA preparations by plasma cholinesterase is conceivable in some cases. The purpose of the present work was to examine this hypothesis.

Methods. The hydrolytic activity of HSA and its inhibition by physostigmine were measured fluorimetrically by monitoring the hydrolysis of the ester substrate moxisylyte. Affinity chromatography was used to separate cholinesterase and HSA. The cholinesterase activity in the eluted fractions was assessed using Ellman's reagent and butyrylthiocholine as substrate.

Results. A significant variation in the esterase-like activity of different albumin batches was observed. This activity was strongly inhibited by physostigmine, a well-known inhibitor of cholinesterase. Affinity chromatography led to a complete separation between HSA and the esterase activity, which was found exclusively in the cholinesterase fraction.

Conclusions. The apparent esterase-like activity of HSA toward moxisylyte and butyrylthiocholine was due to a contamination by cholinesterase. With these substrates, HSA showed a total lack of esterase-like activity.

KEY WORDS: human serum albumin; cholinesterase; esterase-like activity; affinity chromatography.

INTRODUCTION

Serum albumin is the most abundant protein in blood plasma and serves as a carrier and depot protein for a number of endogenous and exogenous compounds. Much attention has therefore been paid to the study of the interaction of drugs and other compounds with serum albumin. Some of these investigations revealed "enzyme-like" activities of the albumin preparations. In several cases, these activities could be attributed to the presence of low levels of contamination of albumin by one or more enzymes (i.e. peptidase, phospholipase) (1). Albumin preparations were also found to hydrolyze some xenobiotic esters. The term "esterase-like" activity of albumin has been introduced to describe these observations. In some cases however, it is still not clear if the activity is intrinsic to the albumin molecule or is in fact due to a low concentration of an esterase impurity in the albumin preparation.

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The catalytic effect of serum albumin on the hydrolysis of *p*-nitrophenyl esters was first noted in 1951, and *p*-nitrophenyl acetate (NPA) is still the most commonly used substrate to determine the esterase-like activity of albumin (2–4). It was demonstrated that NPA acylates the albumin molecule at Tyr-411, localized in binding site II (5). The involvement of Tyr-411 has also been shown for the hydrolysis of various other substrates, such as *p*-nitrophenyl esters of glycine (6), 2,4-dinitrophenyl diethyl phosphate (7) and N-*trans*-cinnamoyl-imidazoles (8).

In the case of acetylsalicylic acid, the participation of albumin in its hydrolysis has been described since 1968 (9) and the lysine residue involved identified as Lys-199 (10). Furthermore, it seems that lysine residues are involved in the β-lactamase activity of albumin (11).

The mechanism of hydrolysis of other esters remains to be elucidated, since it is still unclear whether it occurs according to one of the above mechanisms (i.e., the involvement of the reactive residue Lys-199 or Tyr-411). Indeed, acetylsalicylic acid and some *p*-nitrophenyl esters are quite reactive acylators of nucleophilic sites compared to other esters.

As for the hydrolysis of various esters, a possible contamination of serum albumin by plasma cholinesterase (also known as butyrylcholinesterase; BChE; EC 3.1.1.8) cannot be excluded. This hypothesis was raised in 1962 by Tove (12) and more recently by Whelpton and Hurst (13) while studying the binding of physostigmine to HSA. The efficient hydrolysis of nicotinate esters described by Steiner *et al.* (14) and Salvi *et al.* (15) could be due to contamination by cholinesterase in samples of a commercially available essentially fatty acid free HSA. This may also be the case for the hydrolysis of flestolol, since this drug is known to be hydrolyzed by BChE (16).

The present work examines the hypothesis of a cholinesterase contamination in commercially available albumin preparations. Three different approaches were used. First, the hydrolysis of moxisylyte (Fig. 1) was investigated in the presence of HSA preparations purified by different methods. Second, the inhibitory effect of physostigmine, a well-known inhibitor of cholinesterase, was assessed. Finally, albumin preparations were fractionated using affinity chromatography and the hydrolytic activity of the fractions determined.

MATERIALS AND METHODS

Chemicals

Dextran-coated charcoal, 5,5′-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent), physostigmine hemisulfate, moxisylyte hydrochloride, horse serum cholinesterase and butyrylthiocholine were from Sigma Chemicals (Saint Louis, MO, USA). BCECF (2′,7-bis(carboxyethyl)-5(6)-carboxyfluoresceine) used for the fluorimetric assays was purchased from Calbiochem (La Jolla, CA, USA). Cupric sulfate pentahydrate and ethylenediamine tetraacetic acid (EDTA) were from Fluka (Buchs, CH), potassium sodium tartrate was from Siegfried (Zofingen, CH), Folin-Ciocalten reagent was from Merck (Darmstadt, Germany), procainamide hydrochloride and 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide hydrochloride (DEC) were from Aldrich (Milwaukee, WI, USA). ECH Sepharose 4B was from Amersham Pharmacia Biotech (Uppsala, Sweden). All other chemicals were from the usual

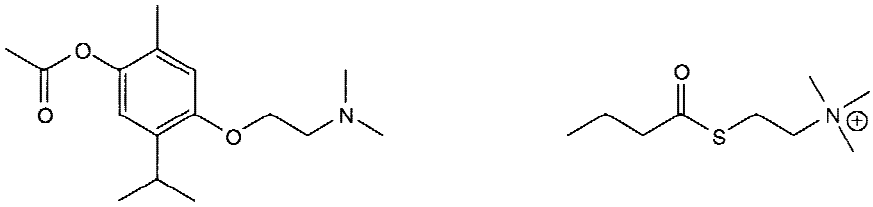

Fig. 1. Chemical structures of moxisylyte and butyrylthiocholine.

commercial sources and were used without further purification.

Human serum albumin preparations from different suppliers were tested to compare their hydrolytic activity:

1. HSA-I: Essentially fatty acid-free human serum albumin A-1887 (≥96%) was obtained from Sigma Chemicals and stored at 2–8°C. According to product information, this HSA is prepared from fraction V (17) and fatty acids are removed by charcoal treatment according to Chen's method (18). Three different batches were tested.
   a) 14H9319 - 1997 - water content 2.6%
   b) 42H9313 - 1995 - water content 6.7%
   c) 118F9311 - 1994 - water content 2.2%.
2. HSA-II: Essentially fatty acid-free, essentially globulin-free HSA (approx. 99%) (A-3782, Sigma). According to product information, this HSA is prepared like A-1887, globulins being then removed by agarose electrophoresis.
3. HSA-III: Essentially fatty acid-free, essentially globulin-free HSA (≥99%) (05418, Fluka). According to product information, this HSA is prepared from fraction V (17), and globulins are precipitated by heat treatment at 67–70°C in the presence of sodium caprylate.
4. HSA-IV: Non-denatured HSA (≥96%) (126654, Calbiochem). According to product information, this HSA is prepared by affinity chromatography; fatty acid content was not indicated.
5. HSA-V: Fraction V (Cohn's) powder (Sigma A-1653); 96–99% albumin.

All solutions were prepared in demineralised and purified water obtained with the Seralpur Pro90 C system (Seral, Renggli, Rotkreuz, CH). Phosphate salts for the buffers came from Fluka, and the solvents of HPLC quality used for the mobile phases were obtained from Romil Chemicals (Loughborough, UK).

Fluorimetric Monitoring of Moxisylyte Hydrolysis

A fluorimetric method was used which allows to monitor the hydrolysis of esters by measuring the rate of proton production; conditions were as published previously (19). The assays were performed with a Perkin-Elmer luminescence spectrometer LS 50B (Perkin-Elmer, UK) in 1 cm, sealed quartz cells (111 QS, Hellma Swiss, Basle, CH). The cells were maintained at 37 ± 0.2°C using a water-bath circulator (Haake D8, Digitana, Lausanne, CH). The working concentrations were: albumin 50 μM, moxisylyte 10^{-3} M, phosphate buffer (pH 7.5) $6.1 \cdot 10^{-3}$ M. Incubation period was 60 min. For inhibition studies, physostigmine concentrations were 20 μM.

The pseudo-first-order rate constants (min^{-1}) were obtained by linear regression from the semi-logarithmic plot of the decay of moxisylyte concentration vs. time.

Ultrafiltration

The ultrafiltration equipment was supplied by Millipore (Le Mont-sur-Lausanne, CH). Ultrafree 0.5 tubes with Biomax membranes (nominal molecular weight limit 5 kDa) were used in a Biofuge 15R centrifuge (Heraeus, Zurich, CH). HSA solutions (50 μM; 0.5 mL) were prepared in phosphate buffer (pH 7.4, 25 mM) and used to dissolve moxisylyte (final concentration: 10^{-4} M). Ten minutes after solution preparation (20 ± 1°C), samples were centrifuged for 8 min at 12,000 g. Filtrates were immediately transferred into glass tubes and kept at 0°C until HPLC analysis. Blanks were prepared to estimate loss by adsorption on membranes and plastic tubes by centrifuging moxisylyte solutions in phosphate buffer.

The HPLC equipment consisted in a LC T-414 pump, a HPLC 432 detector (Kontron Instruments, Zürich-Müllingen, CH), and a HP 3390A integrator (Hewlett-Packard, CA, USA). The column used was a Supelcosil ABZ PLUS column (Supelco, PA, USA) 15.0 cm long, with a diameter of 4.6 mm. The mobile phase was a phosphate buffer (25 mM, pH 5.1)/acetonitrile solution (70:30). Flow rate was 1 mL/min and UV detection was at 274 nm.

Removal of Fatty Acids

To remove fatty acids from albumin, we used a modification (20) of Chen's method (18). A 500 μM HSA solution was prepared. Dextran-coated charcoal (33.5 mg/mL) was added to the solution and the pH lowered to 3.0 by addition of HCl 0.2 M. The solution was then placed on an ice bath and stirred magnetically for 1 h. Charcoal was removed by centrifugation at 19,900 g for 20 min. The clarified solution was brought to pH 7.4 by addition of NaOH 0.2 M and used for the enzymatic assays.

UV Determination of Butyrylthiocholine Hydrolysis

Activities were determined by the general method of Ellman *et al.* (21) using butyrylthiocholine (Fig. 1) as substrate. To detect esterase activity in the fractions separated by affinity chromatography, the reaction mixture consisted of Ellman's reagent (100 μL, 1.5 mM), butyrylthiocholine (100 μL, 150 mM) and 1.0 mL of the eluted fraction. After incubation at 37°C for 10 min, the released thiocholine was measured with a PU 8720 UV/VIS Philips scanning spectrophotometer (Philips Analytical Instruments, Cambridge, GB) using cysteine as standard.

For the Michaelis-Menten kinetic analysis of BChE-

containing fractions, the velocity of hydrolysis was measured at 37°C over a period of 6.0 min after a delay of 5.0 min with a Kontron Uvikon 941 spectrophotometer with final butyrylthiocholine concentrations ranging from 4.6 to 0.15 mM. The Michaelis-Menten constants for unfractionned HSA were measured at a concentration of 4.84 μM and with butyrylthiocholine at concentrations ranging from 4.8 to 0.8 mM. HSA solutions were pre-incubated at 37°C for 1 h. The increase in absorbance at 412 nm was measured during 3.0 min.

The velocities of hydrolysis were determined by linear regression from the initial linear part of the plot of substrate concentration vs. time. The kinetic parameter K_M were calculated by curve fitting according to classical Michaelis-Menten equation using Prism V3.0 (GraphPad Software Inc.).

Determination of Protein Concentrations

The protein content of the eluted fractions was determined according to Lowry *et al*. (22) with HSA-IV as standard. The absorbance of the blue complex was measured at 660 nm with a PU 8720 UV/VIS Philips scanning spectrophotometer.

Cholinesterase Affinity Column

The procainamide-Sepharose 4B affinity gel was synthesized according to the directions of the manufacturer. Typically, a 9.0 mL batch of washed and settled Sepharose 4B was used. Procainamide (305.6 mg, 45 μmol/mL of gel) was coupled to the ECH Sepharose 4B by a 0.1 M solution of DEC by maintaining the pH at 4.5 with 0.1 M HCl for 2 h and then stirring at room temperature for 24 h.

A 4 mL suspensions of the affinity gel was packed in a column (0.9 × 25 cm) and equilibrated with 0.02 M phosphate buffer, pH 6.9, containing 1 mM EDTA (equilibration buffer) (23).

Sample of 1 mL were loaded on the column at a flow rate of 0.6 mL/min. The pump used was an Ismatec Reglo FMI pump (Ismatec, Glattbrugg, CH). After washing with 30.0 mL of the equilibration buffer, elution was performed at a flow rate of 1.2 mL/min with a stepwise increase of ionic strength from 0 to 0.5 M NaCl in the equilibration buffer. Separation was performed at 10°C. Column eluents were collected in fractions of 5-10 mL, in which esterase activity and protein content were determined.

The efficiency of the affinity gel for the BChE enzyme was established by using 0.01 mg of purified horse BChE. The enzyme was eluted with 0.3 to 0.5 M NaCl in the equilibration buffer. The procainamide-Sepharose affinity gel bound horse BChE completely. The enzyme was detected by its activity rather than protein content.

RESULTS

Hydrolysis of Moxisylyte in the Presence of HSA

The results obtained with the various types and batches of albumin are shown in Table I. The half-life of moxisylyte in the presence of 50 μM HSA varied from 0.36 h. to 60 h. HSA-I and HSA-V were particularly active whereas HSA-II, HSA-III and HSA-IV were almost inactive when compared to the chemical hydrolysis of moxisylyte (t½ = 72 ± 1.6 h).

Table I. Esterase-Like Activity of Different Types of HSA toward Moxisylyte[a]

Batches of HSA[b]	Half-life ± SD (n = 3)
HSA-Ia	5.0 ± 0.3 h
HSA-Ib	2.7 ± 0.1 h
HSA-Ic	0.36 ± 0.005 h
HSA-II	60 ± 11 h
HSA-III	46 ± 7 h
HSA-IV	25 ± 4 h
HSA-V	0.68 ± 0.05 h

[a] Conditions: [HSA] 50 μM; [moxisylyte] 10^{-3} M; pH = 7.5; 37°C.
[b] See Materials and Methods for a description of the various batches.

Ultrafiltration

Ultrafiltration assays with HSA-Ia, HSA-II and HSA-IV were performed. The loss by adsorption was determined by ultrafiltrating solutions without HSA, and was estimated to 20 ± 2.5% of the starting concentration (10^{-4} M). Similar losses were measured in the presence of 50 μM HSA (20–29%), leading to the conclusion that no binding occurs in these conditions. Thus, the hydrolysis of moxisylyte in the presence of HSA does not appear to be related to binding.

Removal of Fatty Acids

All albumin types used in this work except HSA-IV were guaranteed to be free from fatty acids. Nevertheless, removal of fatty acids was performed as described. No modification of hydrolytic activity was seen following this treatment, even in the case of HSA-IV. Thus, the low activity of this preparation was not due to the presence of fatty acids.

Inhibition of Moxisylyte Hydrolysis by Physostigmine

Assays in the presence of 50 μM HSA-Ia, HSA-Ib, HSA-Ic or HSA-V and 20 μM physostigmine produced more than 90% inhibition of the esterase activity toward moxisylyte, as shown in Table II.

Separation of BChE and Albumin

Three albumins (HSA-Ib, HSA-Ic and HSA-IV) were eluted through the affinity column. This resulted in a complete separation of the fractions with high protein content (i.e., HSA, fractions 3–7) and the fractions with high esterase activity (19–80), as shown in Fig. 2. The overall recovery calculated was 80–95% of total protein amount.

Table II. Remaining Esterase Activity in HSA Preparations in the Presence of 20 μM Physostigmine[a]

Batches of HSA[b]	Remaining activity (n = 2)
HSA-Ia	9.5%
HSA-Ib	8.5%
HSA-Ic	7.5%
HSA-V	9.0%

[a] Conditions: [HSA]: 50 μM; [moxisylyte]: 10^{-3} M; pH = 7.5; 37°C.
[b] See Materials and Methods for a description of the various batches.

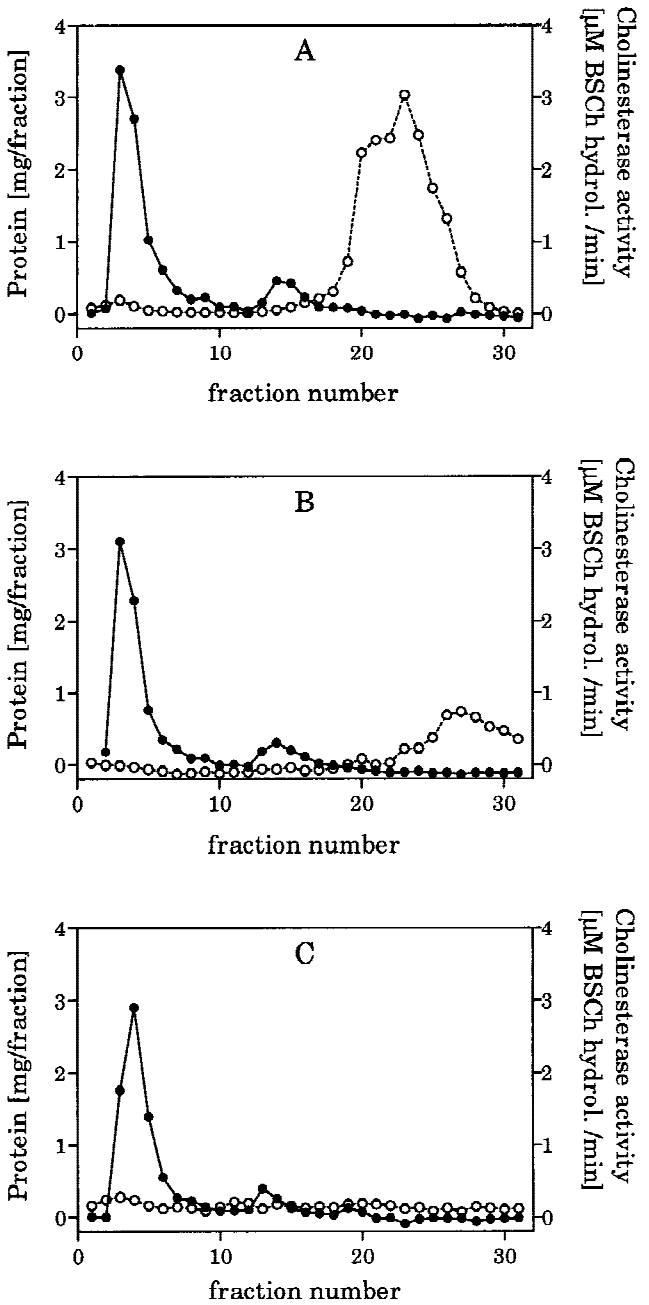

Fig. 2. Procainamide-Sepharose affinity chromatography of various batches of human serum albumin: (A) HSA-Ic, (B) HSA-Ib, and (C) HSA-IV (see Materials and Methods). The traces are the elution profile of protein (●) and BChE activity (○).

The cholinesterase activity was present in exactly the same fractions as obtained with pure horse serum cholinesterase (data not shown). HSA-Ic yielded the highest activities in fractions 19–30 (Fig. 2A). In contrast, HSA-IV had very low activity, and no peak was detected in the cholinesterase fractions (Fig. 2C).

Fractions 20–24 from HSA-Ic were used to establish the Michaelis-Menten kinetics of butyrylthiocholine hydrolysis. This analysis revealed two distinct sites of hydrolysis for moxisylyte, having respectively a high and a low affinity. The Michaelis-Menten parameters obtained as the average of three curves (r^2 = 0.9974–0.9997) were K_{M1} = (26.4 ± 5.6) × 10^{-6} M and K_{M2} = (3.1 ± 1.1) × 10^{-3} M.

DISCUSSION

The esterase activities in the different commercially available types of HSA vary widely. HSA-II displayed no esterase activity toward moxisylyte and butyrylthiocholine, even at concentrations up to 500 μM. The important differences in hydrolytic activity between HSA-Ia, HSA-Ib and HSA-Ic are particularly interesting, since these batches were all prepared according to the same procedure.

A hypothesis that could explain these differences is that moxisylyte binds to some types of albumin more than to others, which would lower its free concentration and enhance its stability. To examine this hypothesis, binding experiments were performed by ultrafiltration. The binding of moxisylyte to HSA was practically nil (between 0–9%). The results obtained showed no correlation between binding to albumin and esterase activity. The difference in hydrolytic activities between HSA-Ia, HSA-Ib and HSA-Ic must therefore be due to the protein solutions themselves.

Another phenomenon able to diminish the esterase-like activity of HSA is the presence of fatty acids (24). All albumin batches used in this work were guaranteed fatty acid-free, except HSA-IV. As a further precaution, removal of fatty acids was performed for HSA-Ia, HSA-Ic and HSA-IV. This treatment had no effect on the hydrolytic activity of HSA preparations toward moxisylyte, even for HSA-IV (data not shown). Hence the presence of fatty acids cannot explain the low activity of HSA-IV.

The failure of the above hypotheses to explain the important differences in hydrolytic activity between our batches of HSA, and the comparatively high activity of HSA-Ic, raised the question of a possible contamination by plasma cholinesterase, as already postulated (13). The important inhibitory effect of physostigmine offers a first evidence for a contamination by BChE. Physostigmine is a reversible, stoechiometric inhibitor of cholinesterases, acting by carbamoylation of the serine hydroxyl group in the active site (25). It is therefore reasonable to postulate that the observed inhibition targets the contaminating cholinesterase, all the more so since in our experiments there was almost complete inhibition with less physostigmine than protein (20 μM vs. 50 μM).

The Michaelis-Menten parameters for hydrolysis of butyrylthiocholine obtained with the active fractions isolated by affinity chromatography of HSA-Ic (K_{M1} = 26.4 ± 5.6 μM; K_{M2} = 3.1 ± 1.1 mM) are similar to the values obtained in preliminary experiments using the same albumin before chromatography (K_{M1} = 26 μM; K_{M2} = 1.9 mM; r^2 = 0.9872). These Michaelis-Menten constants can also be compared with literature values for human cholinesterase. The hydrolysis of butyrylthiocholine by purified human serum cholinesterase at 30°C revealed two sites with K_{M1} = 17.7 μM and K_{M2} = 0.243 mM (26). Our results are also in agreement with a K_M of 27 ± 3 μM for cholinesterase acting on butyrylthiocholine and a K_M of 25 ± 2 μM in the presence of HSA (13). The comparison of the Michaelis-Menten constants for the first site strongly suggests that the hydrolysis of butyrylthiocholine in the presence of HSA is due to a contaminating cholinesterase, as it is unlikely for albumin to have the same affinity than cholinesterase for this substrate. The values for the sec-

ond site are not of the same magnitude, but the results for this low-affinity site may not be reliable or comparable.

Affinity chromatograms (Fig. 2) revealed similar elution profiles, since the three HSA batches examined were separated into two peaks. The major peak was present in fractions 3–7, and the minor peak (about 10% of total proteins) in fractions 13–16. Since the albumin content of the three samples were guaranteed >96%, the second peak has to be albumin, perhaps in polymeric form. No esterase-like activity was found in fractions 3–7 and 13–16. However, there was a esterase activity in fractions 19–30, which decreased in the order HSA-Ic >> HSA-Ib > HSA-IV, in full agreement with the observed half-lives of moxisylyte in the presence of HSA-Ic, HSA-Ib and HSA-IV (Table I). In addition, a very slight activity was observed in fractions 2–4 of HSA-Ic and HSA-IV. This could be due to a non-retained cholinesterase-HSA covalent conjugate (27).

The fact that the two albumin peaks did not show any esterase activity and that this activity could be completely separated from the albumin fractions implies that the observed activity must be due to a contaminant. And since fractions 19–30 had a very little protein content and yet a very high activity, a further implication is that this contaminant must be an enzyme, i.e., a hydrolase. Moreover, this contaminant had the same elution profile and the same affinity toward butyrylthiocholine as pure cholinesterase, confirming that it is indeed cholinesterase.

CONCLUSION

In conclusion, we bring evidence for a cholinesterase contamination in some commercially available HSA preparations. With this information at hand, it becomes critical to use pure HSA (e.g., recombinant, or purified by chromatography or electrophoresis) when studying the hydrolytic activity of this protein. Similarly, contaminated samples of HSA may yield misleading results when studying the binding affinity of pharmaceutical esters able to undergo cholinesterase-catalyzed hydrolysis.

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