TABLE 1

| | No. of cases | Epidemic Rickettsial Antigen | Dil. of serum | Endemic Rickettsial Antigen | Dil. of serum |
|---|---|---|---|---|---|
| Endemic Typhus | | | | | |
| 43 cases | 5 | negative | | 4 plus | 1:12 |
| | 12 | negative | | 4 plus | 1:24 |
| | 6 | negative | | 4 plus | 1:48 |
| | 9 | negative | | 4 plus | 1:96 |
| | 2 | negative | | 4 plus | 1:192 |
| | 1 | 4 plus | 1:6 | 4 plus | 1:96 |
| | 1 | 4 plus | 1:6 | 4 plus | 1:192 |
| | 4 | 4 plus | 1:12 | 4 plus | 1:48 |
| | 2 | 4 plus | 1:12 | 4 plus | 1:96 |
| | 1 | 4 plus | 1:24 | 4 plus | 1:384 |
| Epidemic Typhus | | | | | |
| 29 cases | 2 | 4 plus | 1:12 | negative | |
| | 1 | 4 plus | 1:24 | negative | |
| | 1 | 4 plus | 1:48 | negative | |
| | 2 | 4 plus | 1:96 | negative | |
| | 13 | 4 plus | 1:192 | negative | |
| | 6 | 4 plus | 1:384 | negative | |
| | 1 | 4 plus | 1:768 | negative | |
| | 1 | 4 plus | 1:96 | 4 plus | 1:24 |
| | 1 | 4 plus | 1:192 | 4 plus | 1:48 |
| | 1 | 4 plus | 1:192 | 4 plus | 1:96 |
| Brill's disease | | | | | |
| 23 cases | 4 | 4 plus | 1:12 | negative | |
| | 2 | 4 plus | 1:24 | negative | |
| | 4 | 4 plus | 1:48 | negative | |
| | 2 | 4 plus | 1:48 | 4 plus | 1:6 |
| | 1 | 4 plus | 1:48 | 4 plus | 1:12 |
| | 1 | 4 plus | 1:96 | 4 plus | 1:48 |
| | 1 | 4 plus | 1:96 | 4 plus | 1:12 |
| | 2 | 4 plus | 1:384 | 4 plus | 1:48 |
| | 3 | 4 plus | 1:768 | 4 plus | 1:192 |
| | 1 | 4 plus | 1:960 | 4 plus | 1:96 |
| | 1 | 4 plus | 1:960 | 4 plus | 1:384 |
| | 1 | 4 plus | 1:1436 | 4 plus | 1:192 |

usually occurs in a family, no vector or reservoir of the virus has been found, and the virus isolated from the blood of the patient has the characteristics observed for the epidemic strain. On the basis of these observations Zinsser[6] advanced the theory that Brill's disease represented a recrudescence of an old attack of typhus fever. Our observations bring serological evidence to substantiate this point of view.

In 23 cases of Brill's disease examined all showed a positive complement fixation with an epidemic rickettsial antigen. In 10 cases there was fixation with an epidemic rickettsial antigen and no fixation with an endemic rickettsial antigen. In 13 cases there was some cross fixation but in all instances where this occurred the titre obtained was higher with an epidemic antigen. The pattern of fixation in this disease resembles that obtained in epidemic typhus fever.

Absorption tests were performed on specimens of serum from Brill's disease where cross fixation had occurred. An endemic rickettsial antigen removed all the endemic antibody with slight effect upon the titre of epidemic antibody. On the other hand, a similar treatment of the serum with an epidemic rickettsial antigen resulted in the removal of both the epidemic and endemic antibody; no selectivity of absorption was observed. These results would indicate that the endemic rickettsial antigen pattern was different from that of the antigenic pattern of the epidemic strain. The removal unselectively of both endemic and epidemic antibodies by the epidemic antigen suggests that the epidemic antigen may be a more complete or complex antigen than the endemic antigen.

The implication of the results obtained in Brill's disease on the epidemiology of typhus fever is great. They would indicate that mild cases of epidemic typhus actually exist in the United States. The disease is not transmitted from person to person in this country simply because the louse vector is not present. Furthermore, these results indicate that one attack of typhus does not confer a lifelong immunity as is generally believed. The virus is probably harbored in the body and when the resistance is lowered the virus multiplies and induces a mild attack of the disease. If these cases should occur in a louse-infested community the disease might readily spread from person to person. The observations on Brill's disease strongly suggest that man serves as the reservoir for epidemic typhus between outbreaks just as the rat does in endemic typhus.

The complement fixation test now provides a tool with which surveys of the prevailing types of typhus in a region can be determined. This procedure has been applied and endemic typhus has been discovered in Jamaica and epidemic typhus in a South American country. These surveys are now being continued in other countries.

The author wishes to thank Captain S. C. Bukantz, Lt. K. Wertman, Lt. L. Pillemer and Miss N. Rogers for their assistance, as well as Dr. E. Schoenbach for having provided the specimens of serum from cases of Brill's disease.

HARRY PLOTZ

ARMY MEDICAL SCHOOL,
WASHINGTON, D. C.

[6] H. Zinsser, *Am. Jour. Hyg.*, 20: 513, 1934.

## A STUDY OF CRYSTALLINE BEEF LIVER CATALASE DRIED IN THE FROZEN STATE[1]

### INTRODUCTION

THE drying of proteins by the lyophile process is assuming increasing importance in research and in preparing such material as dried plasma or dried pollen extracts for practical use. There is a tendency to assume that in drying a quickly frozen protein preparation by subjecting it to a high vacuum, no change such as denaturation occurs. The following observations show that this is not necessarily true.

[1] From the Department of Biochemistry and Pharmacology and the Department of Medicine, The University of Rochester School of Medicine and Dentistry, Rochester, New York.

They also indicate that lyophilization might be used in selected cases as a means to study the process of protein denaturation. Finally they make it appear possible that new information about the chemistry of an enzyme molecule may be obtained from a study of the lyophilized material.

## EXPERIMENTAL

Twice-crystallized beef liver catalase,[2] consisting of a suspension of plates and prisms in water, was quickly frozen in a thin layer by cooling in an acetone-dry ice bath. Then a vacuum pump was attached and the preparation was removed from the cold bath and left connected with the pump until drying was complete. The frozen suspension did not melt at any time during the drying. A separate sample that was heated in the oven to 100° C for two hours did not change in weight.

The dried powder was suspended in water and was examined microscopically. Large crystals of the prism type were disintegrated, but the small crystals of this type retained their original form. The plates were fragmented. The dried preparation though insoluble in water was easily soluble in excess M/1,000 disodium phosphate. In concentrated suspensions it became soluble if sufficient disodium phosphate was added to bring the pH to 8.0. By this procedure, concentrated solutions were obtained which showed a slight opalescence and gave a very strong qualitative test for catalase. The usual three-banded catalase spectrum was present. Quantitative determinations however showed that the activity per dry weight of the material was only about one third that of undried crystalline beef liver catalase,[2] since the *Kat. f.* was about 11,000.

The solubility of the dried preparation differs from that of the undried material, since it is only slightly soluble below pH 8 in the presence of 10 per cent. NaCl. Moreover, it has not been possible to crystallize the dried material, either by the ammonium sulfate method[3] or by the dialysis method.[2] This indicates that a change has been brought about in the enzyme molecule, such as a mild denaturation. It is of interest in this connection that lyophilized catalase keeps quite well in a desiccator, but if left exposed to the atmosphere it gradually loses its solubility during a period of about two months and finally becomes quite insoluble and inactive.

The absorption spectrum of dried catalase was compared with that of undried catalase by Mr. Ralph Brauer of this Department of Biochemistry and Pharmacology, using a visual spectrophotometer. The two samples were adjusted in concentration so that the intensities of the bands at 630 mμ were equal. We have not yet determined whether following this procedure the concentrations on a dry-weight basis are equal. The two absorption spectra do not appear to differ significantly, as shown in Fig. I.

Dried catalase is inhibited by $NH_2OH$ and HCN, as is undried catalase. In the presence of $NH_2OH$, the addition of a little hydrogen peroxide causes the appearance of the two-banded spectrum reported by Keilin and Hartree[4] for undried catalase. Dried

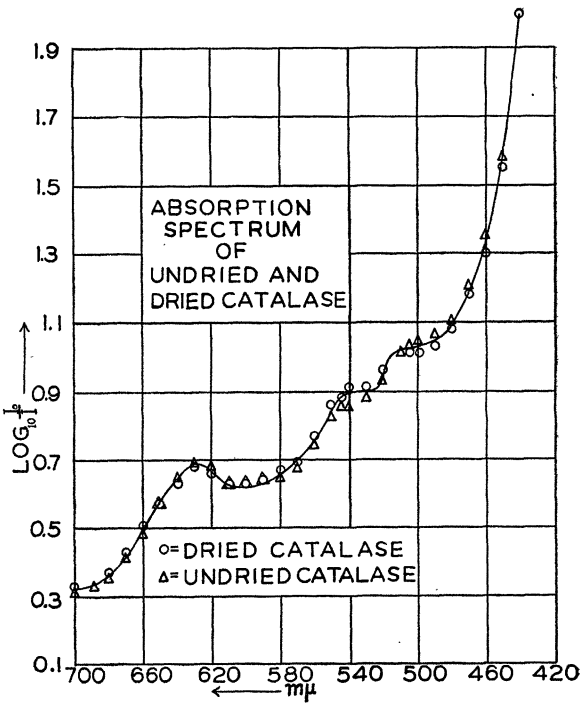


FIG. 1

catalase gives the same bands with HCN, as does undried. Thus the behavior of the dried catalase towards $NH_2OH$ and HCN seems to be identical with that of undried catalase.

One of the peculiarities of ordinary undried catalase is that the hematin iron is not reduced by $Na_2S_2O_4$ alone. However, Zeile *et al.*[5] reported that they were able to reduce the catalase-$H_2S$ compound with $Na_2S_2O_4$ after excess hydrogen sulfide had been removed by passing an inert gas through the solution. If oxygen were admitted and the solution shaken, the reduced catalase was oxidized and the original spectrum returned.

We have found that our dried catalase when in solution is reduced by adding $Na_2S_2O_4$ buffered at pH 7.5, as is shown by the two-banded spectrum

[2] J. B. Sumner and A. L. Dounce, *Jour. Biol. Chem.*, 127: 439, 1939.

[3] J. B. Sumner and A. L. Dounce, *Jour. Biol. Chem.*, 121: 417, 1937.

[4] D. Keilin and E. F. Hartree, *Proc. Roy. Soc. London*, B 124: 397, 1938.

[5] K. Zeile, G. Fawaz and V. Ellis, *Zeits. Physiol. Chem.*, 263: 181, 1940.

which appears, with a strong band at 560 mμ and a weaker and narrower band at 594 mμ. The dithionite can not be replaced by hydrogen sulfide or by a mixture of sulfite and bisulfite of neutral reaction. The two-banded spectrum given by reducing dried catalase with $Na_2S_2O_4$ is quite different from the two-banded hemochromogen-type spectrum which one obtains by adding $Na_2S_2O_4$ to a dried catalase solution which has been boiled. The latter shows bands at 560 mμ and 528 mμ, if light is made to pass through the precipitate which consists of completely heat-denatured catalase. This material of course is inactive towards hydrogen peroxide.

The two-banded spectrum of dried catalase treated with $Na_2S_2O_4$ is changed back to the original catalase spectrum on bubbling oxygen for a few minutes through the preparation, while the addition of a small amount of hydrogen peroxide causes the spectrum to revert instantly to the original catalase spectrum. The latter observation does not argue in favor of the mechanism of reaction of catalase with hydrogen peroxide proposed by Keilin and Hartree,[4,6] in which the iron was thought to be reduced by hydrogen peroxide and reoxidized by molecular oxygen. Dried catalase treated with $Na_2S_2O_4$ is qualitatively as active as dried catalase itself.

The behavior of dried catalase towards $Na_2S_2O_4$, which is probably its most interesting attribute, is summarized in Table 1, together with the corresponding behavior of undried catalase and boiled, dried catalase.

TABLE 1

| Catalase Employed | Effect of Adding $Na_2S_2O_4$ |
|---|---|
| Undried Crystalline Beef Liver Catalase | No Spectral Change |
| Dried Crystalline Beef Liver Catalase | A Two-Banded Spectrum Produced. Bands at 594 mμ and 560 mμ |
| Boiled, Dried Crystalline Beef Liver Catalase | A Two-Banded Hemochromogen Type Spectrum Produced. Bands at 560 mμ and 528 mμ |

## DISCUSSION

A possible explanation for the reducibility of dried catalase is that whatever change has been produced in the catalase molecule by drying includes a sufficient loosening of the linkage between iron and protein to permit $Na_2S_2O_4$ to reduce the iron. The question may be raised as to whether all of the lyophilized catalase has been changed in some way, or whether two thirds has been changed and one third left unaffected. To settle this point with certainty, much work of a physico-chemical nature might be necessary, but the behavior towards $Na_2S_2O_4$ makes it appear probable that none of the enzyme has escaped at least some change. Otherwise, one would expect to obtain mixed spectra of unchanged catalase and $Na_2S_2O_4$-treated catalase. At the present time it seems probable that the change produced in catalase by drying in the frozen state is of the nature of a very mild denaturation.

Another question of importance is whether the change in the enzyme molecule really occurs during the drying or during subsequent solution. The loss in activity and development of insolubility, which evidently is produced by atmospheric moisture acting over a period of time, indicates that at least part of the change may well occur during solution of the enzyme. It is even possible that eventually one might find a way to dissolve the enzyme so as to obtain it in the original condition.

Finally, one might wonder whether drying amorphous instead of crystalline catalase would give different results. This has not yet been investigated, but a solution of recrystallized urease was frozen and dried to an amorphous powder which was found to be completely water-soluble immediately after drying. It showed a high qualitative activity; quantitative determinations were not run. On standing exposed to the atmosphere the material gradually became insoluble and lost most of its activity during a period of about three months.

It should perhaps be noted that the work reported here deals with highly purified proteins of relatively high molecular weight,[7,8] so that any conclusions which one may wish to draw do not necessarily apply to protein mixtures or to certain low-molecular weight proteins like heart-muscle cytochrome C, which can be dried without apparent change.

## SUMMARY

Crystalline beef liver catalase has been dried by the lyophile process, and the properties of the dried preparation have been compared with those of undried crystalline beef liver catalase. The dried material is not crystallizable, possesses about one third the activity per dry weight of undried crystalline beef liver catalase, and in contrast to the undried material, its hematin iron can be reduced by $Na_2S_2O_4$ alone. It is thought that the process of drying, or the subsequent solution of the dried material, produces some change in the molecular structure such as a mild denaturation.

ALEXANDER L. DOUNCE
JOE W. HOWLAND

[6] D. Keilin and E. F. Hartree, *Nature*, 144: 787, 1939.

[7] J. B. Sumner and N. Gralén, *Jour. Biol. Chem.*, 125: 33, 1938.

[8] J. B. Sumner, N. Gralén and I.-B. Eriksson-Quensel, *Jour. Biol. Chem.*, 125: 37, 1938.