sis of various aliphatic acyl and aminoacyl mercaptans of glutathione (GSH) and N-acetylcysteine.

Of the following acyl mercaptans used as substrates S-acetyl-, S-crotonyl-, S-L-alanyl-, S-acetylglycyl-GSH and S-N-diacetylcysteine were prepared by modifications of known procedures.[6-10] S-L-α- and S-L-γ-glutamyl-GSH were prepared by reacting α- or γ-carbobenzyloxy-thioglutamic acid with GSH and subsequent removal of the carbobenzyloxy moiety with hydrogen bromide in phenol-acetic acid.

The acyl mercaptans were determined as hydroxamic acids after reaction with hydroxylamine. The data in the table record the specific activities, with the various acyl mercaptans as substrates, of several ammonium sulfate fractions of a liver extract. The ratio of specific activity with any substrate except S-L-α-glutamyl-GSH to the specific activity with S-acetyl-GSH as the substrate changed from one fraction to another. The table lists those fractions in which the highest and lowest ratios were found. The ratios of rates of hydrolysis of S-L-α-glutamyl- and S-acetyl-GSH, respectively, varied with the different enzyme fractions over a narrower range than those of any of the other substrates. The present data do not exclude the possibility that α-glutamyl- and acetyl-GSH are hydrolyzed by the same enzyme while the hydrolysis of the other acyl mercaptans appears to be catalyzed by enzymes distinct from each other as well as from the S-acetyl-GSH thioesterase. Because of its instability comparative data on the enzymatic hydrolysis of S-L-γ-glutamyl-GSH could not be obtained. The specificity of the thioesterases was not only directed towards the acyl moiety but also towards the mercaptan as demonstrated by the different rates of hydrolysis for S-acetyl-GSH and S-N-diacetylcysteine in different fractions.

Our observations do not indicate whether the thioesterases communicated from other laboratories[11,12,13] while this work was in progress, are different from some of those reported here.

It will be of particular interest to ascertain the activity of these enzymes in hydrolyzing amide and O-ester linkages and in catalyzing the transfer of the acyl and aminoacyl radicals to acceptors other than water.

TABLE I

THIOESTERASE ACTIVITY OF FRACTIONS OF LIVER (OX)

$S.A._1$ specific activity of ammonium sulfate fraction with substrate listed; $S.A._2$ specific activity with S-Acetyl-GSH as substrate. All values corrected for non-enzymatic splitting of thioesters.

	$S.A._1$	$S.A._2$	$S.A._1/S.A._2$	Fractions
S,N-Diacetyl-cysteine	0	15.7	0	C
	7.3	11.1	0.66	A
S-Acetylglycyl-GSH	16.7	11.1	1.5	A
	79.0	15.7	4.9	C
S-Crotonyl-GSH	8.0	21.5	0.37	Orig.
	14.8	15.7	0.94	C
S-L-Alanyl-GSH	12.3	11.2	1.1	B
	39.3	15.7	2.5	C
S-α-Glutamyl-GSH			1.4–1.6	(9 fractions)

Of S-alanyl-GSH 5 μM. and of the other substrates 2.5 μM. were incubated with 0.15 to 0.5 mg. of protein and 50 μM. tris-hydroxymethyl-aminomethane (pH 7.6) in a total volume of 1 ml. of 5 μM. S-L-α-glutamyl-GSH were incubated with 50 μM. acetate (pH 5.5) and the same amounts of protein. Incubation 30′ at 30°. Orig. = extract of acetone-dried powder with 0.02 M K_2HPO_4 (pH 7.4). Fraction A was obtained between 28–36% $(NH_4)_2SO_4$ saturation of original extract. Fractions B and C were obtained by refractionation of the combined 0–28 and 36–64% fractions at 0–28, and 36–48% $(NH_4)_2SO_4$ saturation, respectively.

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THE ROLE OF MOLYBDENUM AND FLAVIN IN HYDROGENASE

Sir:

Clostridium pasteurianum, an obligate anaerobic bacterium that fixes elemental nitrogen contains relatively large amounts of hydrogenase. Cell-free extracts of this enzyme were prepared by subjecting the organism to sonic vibrations and the enzyme was purified by serial application of the following procedures: separation of the particulate fraction by high speed centrifugation, removal of impurities first with protamine sulfate, followed by zinc hydroxide gel, and finally fractional precipitation with ammonium sulfate. The enzyme was not sedimented during a thirty-minute exposure to 144,000 × gravity.

The activity of the enzyme was followed manometrically—the assay being based on the oxidation of molecular hydrogen by methylene blue. At the highest purity level obtained, 1 mg. of protein N catalyzed the oxidation of 750,000 μl. of hydrogen per hour at 30°. Purified preparations were stable for two weeks when stored at 2°, under hydrogen, in presence of 30–40% ammonium sulfate and at pH 6–6.5. The enzyme was inactivated by exposure to oxygen.

While crude preparations of the enzyme readily catalyzed the oxidation of hydrogen by mammalian cytochrome c, this property was either reduced or abolished in the purified preparation. However, full activity could be restored by addition to the purified extracts of molybdenum in the form of MoO_3. Molybdenum could not be replaced by Mn^{++}, Mg^{++}, Fe^{++}, Fe^{+++}, Cu^{+}, Cu^{++}, Co^{++}, $WO_4^{=}$ or VO_3^{-}. The presence of inorganic phosphate was required for the catalysis of cytochrome c reduction by molybdenum. The dissociation of molybdenum from the enzyme takes place throughout the purification procedure. However, the presence of the metal in bound form is demonstrable at all levels of purity.

When the absorption spectrum of the enzyme ox-

idized by oxygen is subtracted from that of the enzyme reduced with hydrogen a difference spectrum such as shown in Fig. 1A is obtained. The two banded difference spectrum with maxima at 450 and 390 mμ resembles closely that of riboflavin. Indeed the presence of FAD[1] in boiled extracts of the enzyme is demonstrable by spectroscopic and enzymatic tests. That the flavin is implicated in the action of hydrogenase can be shown by the following experiment. If the cuvette containing the enzyme reduced by hydrogen is evacuated, then only a negligible difference is observed between the spectra of the oxidized enzyme and the enzyme in the evacuated cuvette (Fig. 1B). This result has been interpreted to mean that the interaction of hydrogen with flavin is reversible[2] and thus at low pressures of hydrogen reduced flavin is oxidized to hydrogen gas and oxidized flavin.

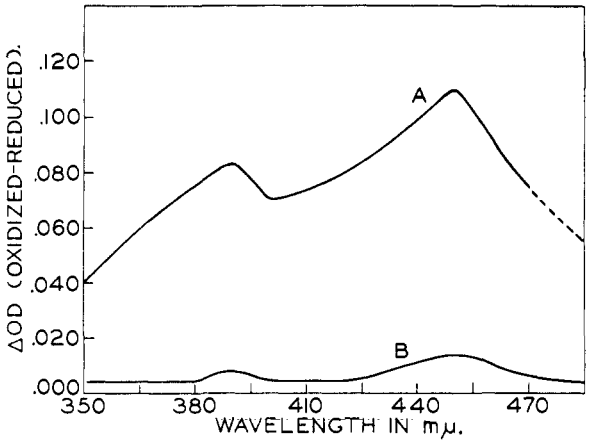

Fig. 1.—The difference spectrum (oxidized–reduced) of hydrogenase: 3 ml. of a purified preparation containing 1.8 mg. protein per ml. was reduced with hydrogen and oxidized with oxygen. The difference in optical density is recorded in curve A. In curve B the same amount of enzyme was reduced again with hydrogen, and then the hydrogen carefully removed by evacuation, and the difference in optical density between the oxidized spectrum and that obtained at low hydrogen pressure plotted.

The purified enzyme is not completely precipitated in 70% saturated ammonium sulfate. When such a solution is first clarified by centrifugation and then dialyzed for 4 hours at *p*H 7.5, the activity of the enzyme with either methylene blue and cytochrome *c* as electron acceptors is very low (Curve A of Fig. 2). The activity of such an enzyme toward cytochrome *c* can be restored by the addition of *both* FAD or a boiled extract of the enzyme (but not FMN), and molybdenum (Curve B, Fig. 2). No restoration of activity occurs with either FAD (or boiled extract), or Mo, if they are added singly.

The presence of Mo is required only for the oxidation of hydrogen by one electron acceptor like cytochrome *c* but not for the oxidation of hydrogen by methylene blue. Furthermore, the presence of phosphate is a requirement only for the metal-catalyzed oxidations of hydrogenase. Thus the pattern

(1) FAD is flavinadenine dinucleotide; FMN is riboflavin-5'-phosphate.

(2) D. E. Green and L. H. Stickland, *Biochem. J.*, **28**, 898 (1934).

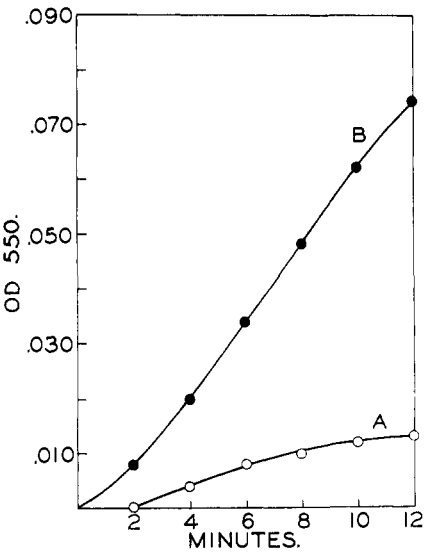

Fig. 2.—Restoration of activity to the supernatant from 70% $(NH_4)_2SO_4$ (0.120 mg. protein per test) by addition of FAD and molybdenum: tests were performed with Pyrex cells with a Thunberg attachment. At zero time the enzyme, incubated 15 minutes with FAD or a boiled extract in an atmosphere of hydrogen, was tipped into 3 ml. of *M*/15 phosphate buffer at *p*H 6.8 containing 1 mg. of cytochrome *c* and 23 γ of molybdenum. The contents were the same for the blank, except that the atmosphere was air.

for hydrogenase resembles closely that observed for two other molybdoflavoproteins, *viz.*, milk xanthine oxidase and liver aldehyde oxidase.[3,4]

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EVALUATION OF SCHERAGA AND MANDELKERN'S SHAPE FACTOR FOR BOVINE SERUM ALBUMIN[1]

Sir:

It has been customary to interpret the hydrodynamic properties (*e.g.*, diffusion, sedimentation, viscosity) of protein molecules in terms of an equivalent hydrated ellipsoid. The results have always been somewhat ambiguous because these hydrodynamic properties are affected both by hydration and by shape: a satisfactory separation of these factors has not been possible. Recently Scheraga and Mandelkern[2] attempted to resolve this ambiguity by combination of two different hydrodynamic properties: they showed that the function

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(2) H. A. Scheraga and L. Mandelkern, THIS JOURNAL, **75**, 179 (1953).