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.

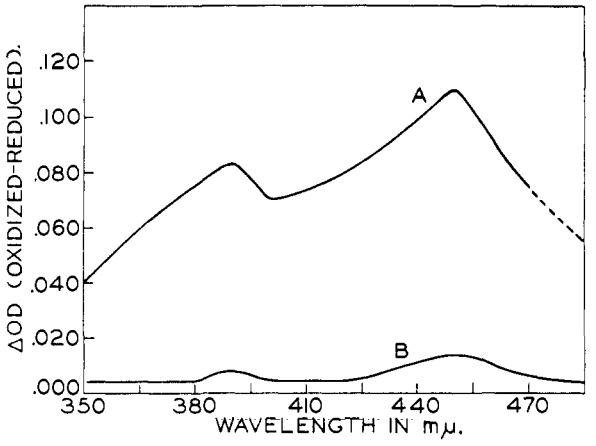

Fig. 1.—The difference spectrum (oxidized–reduced) of hydrogenase: 3 ml. of a purified preparation containing 1.8 mg. protein per ml. was reduced with hydrogen and oxidized with oxygen. The difference in optical density is recorded in curve A. In curve B the same amount of enzyme was reduced again with 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).

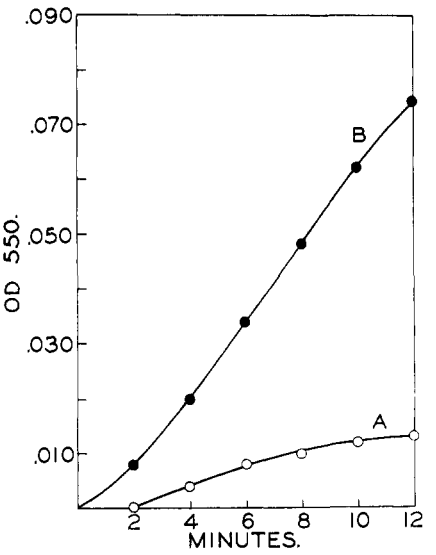

Fig. 2.—Restoration of activity to the supernatant from 70% $(NH_4)_2SO_4$ (0.120 mg. protein per test) by addition of FAD and molybdenum: tests were performed with Pyrex cells with a Thunberg attachment. At zero time the enzyme, incubated 15 minutes with FAD or a boiled extract in an atmosphere of hydrogen, 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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(3) B. Mackler, H. R. Mahler and D. E. Green, *J. Biol. Chem.*, in press.

(4) H. R. Mahler, B. Mackler and D. E. Green, *ibid.*, in press.

(5) Supported by a grant from the Wisconsin Alumni Research Foundation.

(6) Supported by a grant from the National Institutes of Health.

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

(1) This work is supported by grants from the Research Corporation, The National Science Foundation and The National Institutes of Health, Public Health Service.

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

β, defined as $Ns[\eta]^{1/3}\eta/M^{2/3}(1 - \bar{v}\rho)$ or $D[\eta]^{1/3}M^{1/3}\eta/kT$, is independent of any assumption concerning hydration, depending only upon the shape of the hydrodynamic particle. They suggested that the value of β be used to define a rigid ellipsoid equivalent to the protein hydrodynamic particle.

We have recently determined in this laboratory the intrinsic viscosity of isoionic bovine serum albumin. We found, at 25° and independent of ionic strength $[\eta] = 0.038$. We have calculated the same quantity from data of Yang and Foster,[3] and obtained $[\eta] = 0.036$.

Recent accurate determinations are available for all other physical constants required to compute β for bovine serum albumin. The molecular weight[4] is 65,000 ± 2000, the sedimentation constant $(s_{20,w})$[5] is $(4.30 \pm 0.03) \times 10^{-13}$, the diffusion constant $(D_{20,w})$[6] is $(6.15 \pm 0.02) \times 10^{-7}$, $\bar{v}$ is 0.734 ± 0.003.[7] Combination with the mean of Foster's and our value of $[\eta]$, with an assigned uncertainty of ±0.002, then yields for Scheraga and Mandelkern's shape factors, $\beta = (2.04 \pm 0.06) \times 10^6$ (using $d_{20,w}$) or $(2.01 \pm 0.12) \times 10^6$ (using $s_{20,w}$). It is thus highly probable that the value of β lies outside the permissible range for rigid ellipsoids of revolution ($\beta \geqslant 2.12 \times 10^6$)[2]: it is impossible to find such an ellipsoid with the same value of β as the hydrated serum albumin molecule.

It is worthwhile to examine the reason for this failure of the method of Scheraga and Mandelkern.

A sphere provides the least possible surface of contact with the solvent. Any deviation whatever from spherical shape, at constant volume (*i.e.*, hydration), will lead to an increase in this surface, and, hence, to an increase in $[\eta]$ and in frictional constant and to a decrease in D and s. It is thus always possible to find an ellipsoid with the same value of $[\eta]$, D or s as a particle of the same volume deviating from spherical shape in some other way.

The same is not true for the product $s[\eta]^{1/3}$ or $D[\eta]^{1/3}$ occurring in Scheraga and Mandelkern's β. If, for a given deviation from spherical shape, the increase in $[\eta]^{1/3}$ is greater than the decrease in s or D, then β will increase; if, however, the decrease in s or D is greater than the increase in $[\eta]^{1/3}$, then β will decrease. Thus distortion of a sphere to form a prolate ellipsoid may change β in one direction; distortion to give some other geometric shape may change it in the opposite direction.[8] There is therefore no reason to believe that β values for other than ellipsoidal particles need fall within the range applicable to ellipsoids.

It should also be pointed out that protein molecules are not necessarily rigid, and that this, too, will have as yet unpredictable effects on all hydrodynamic properties.

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(3) J. T. Yang and J. F. Foster, THIS JOURNAL, **76**, 1588 (1954). We are grateful to Dr. Foster for providing us with detailed experimental data in advance of publication.

(4) (a) J. M. Creeth, *Biochem. J.*, **51**, 113 (1952); (b) S. N. Timasheff, personal communication.

(5) Six independent determinations, most of them quoted by S. Shulman, *Arch. Biochem. Biophys.*, **44**, 230 (1953). To combine these values with the intrinsic viscosity at 25° we have used the fact that $s\eta/(1 - \bar{v}\rho)$ is independent of temperature.

(6) Ref. 4a; we have made use of the temperature-independence of the ratio $d\eta/T$.

(7) M. O. Dayhoff, G. E. Perlmann and D. A. MacInnes, THIS JOURNAL, **74**, 2515 (1952). Within the uncertainty given, $\bar{v}_{20} = \bar{v}_{25}$.

(8) The dependence of β on actual geometric shape is already observed when one compares prolate and oblate ellipsoids.[4] For the former β increases with increasing axial ratio; for the latter, on the other hand, it is essentially independent of axial ratio. The quantities D, s and $[\eta]$, on the other hand, depend strongly on axial ratio both for prolate and oblate ellipsoids.

INACTIVATION OF INSULIN BY RAT LIVER EXTRACTS[1]

Sir:

The inactivation of insulin by rat tissue extracts has been reported to occur to the greatest extent with extracts of the liver.[2,3] In these earlier investigations the mode of this inactivation was not elucidated.

In the present report, evidence is presented which indicates that insulin is inactivated mainly by proteolysis. Furthermore, the enzyme system responsible for this action has an apparent specificity which warrants further investigation.

The preparation of the liver extract and the conditions of incubation were essentially the same as described earlier by Mirsky and Broh-Kahn.[3] A modification of this procedure was the addition of a trace amount of insulin-I^{131} [4] to the substrate of amorphous insulin.[5] All incubations were terminated by the precipitation of proteins with the addition of trichloroacetic acid (TCA) to a final concentration of less than 10%. The amount of radioactivity in the supernatant and the precipitate was determined with a well-type γ-counter. In some of the experiments, the supernatant was assayed for nitrogen, by the Kjeldahl method, and for total radioactivity. In these experiments, control incubations of extract alone were run. The results, shown in Fig. 1, demonstrated that with increasing incubation time there was good correlation between the

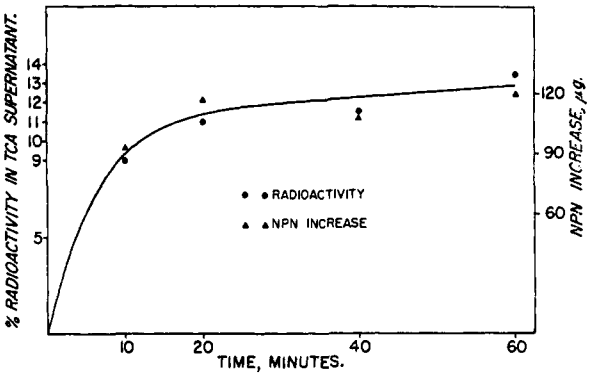

Fig. 1.—Changes with time in NPN and % radioactivity in TCA supernatant: Incubation of 3 mg. amorphous insulin and tracer amount of insulin-I^{131} with extract from 1 g. rat liver.

(1) Supported by grants from Atomic Energy Commission, United States Public Health Service, Eli Lilly and Co., and Initiative 171, State of Washington.

(2) A. A. Shmidt and R. L. Saatchian, *Zhur. Eksptl. Biol. i. Med.*, **11**, 42 (1929).

(3) I. A. Mirsky and R. H. Broh-Kahn, *Arch. Biochem.*, **20**, 1 (1949).

(4) Insulin-I^{131} obtained from Abbott Laboratories.

(5) Amorphous insulin, assaying 19 u/mg., kindly supplied by Drs. O. K. Behrens and C. W. Pettinga of Eli Lilly and Co.