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.[2] 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

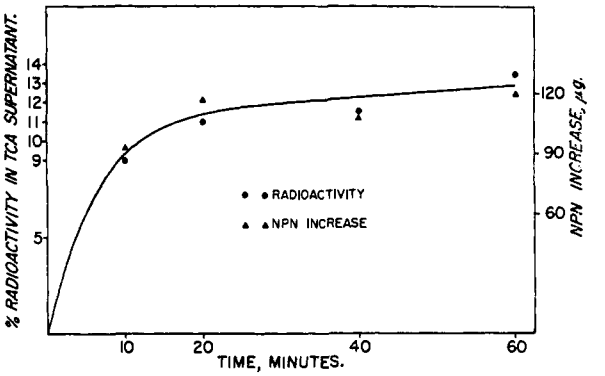

Fig. 1.—Changes with time in NPN and % radioactivity in TCA supernatant: Incubation of 3 mg. amorphous insulin and tracer amount of insulin-I^{131} with extract from 1 g. rat liver.

(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. Ekspil. 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.

rise in non-protein nitrogen (NPN) and the increase in % of radioactivity in the TCA supernatant. This observation is presumptive evidence that insulin was being proteolytically degraded by the extract and that the determination of % of radioactivity in the TCA supernatant is a valid method for detection of this proteolysis.

In order to relate proteolysis with the inactivation of the biological action of insulin, the radioactive insulin assay was compared directly with the rabbit hypoglycemia test of Mirsky and Broh-Kahn.[3] In this case, each of the incubation mixtures was divided into two parts and assayed by both methods. With increased proteolysis, decreased biological activity of the incubated insulin was demonstrated. As a further comparison, studies of various inhibitors with known effects on the insulin-inactivating action of liver extract were repeated. In these cases, only the assay for radioactivity was carried out. The results with these inhibitors were in good agreement with the earlier work in which the rabbit assay was used.[3]

Studies on the specificity of the enzyme system have been made. The results of an experiment using the technique of substrate competition are shown in Fig. 2. Although amorphous insulin in excess clearly depressed the rate of degradation of insulin-I^{131} in a given amount of extract, the addition of the same weight of α-lactalbumin, human serum albumin or casein had negligible effects. This indicated that of these four proteins, only amorphous insulin effectively competed with insulin-I^{131} in this system. In order to substantiate this view, these four proteins were incubated with liver extract for 30 min. and increases in NPN were determined. Only with insulin as substrate was there a measurable increase in NPN over the control incubation mixture. These experiments demonstrate that the insulin-inactivating enzyme system has some degree of specificity.

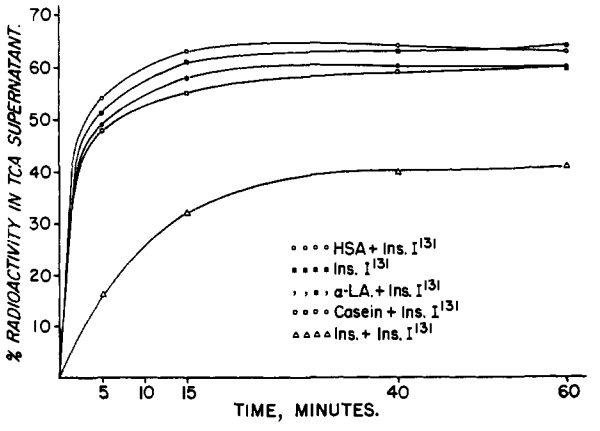

Fig. 2.—Changes with time in % radioactivity in TCA supernatant: Incubation of tracer amount of insulin-I^{131} alone and with 0.125 mg. of specified proteins; Extract used obtained from 0.3 g. rat liver.

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MECHANISM OF ENZYMATIC CARBON DIOXIDE FIXATION INTO OXALOACETATE

Sir:

This report concerns the mechanism of the enzymatic conversion of phosphoenol pyruvate (PEP) and carbon dioxide into oxaloacetate (OAA) and inorganic phosphate. Such a reaction was first obtained with spinach leaf preparations by Bandurski and Greiner.[1] The present studies were made with dialyzed extracts of wheat germ. By conducting the reaction in a medium of D_2O, it has been possible to show that it is the keto form of oxaloacetate, (and not an enol) which results from the carbon dioxide fixation reaction. The mechanism is therefore similar to the reverse of the mechanism demonstrated by Steinberger and Westheimer[2] for the decarboxylation of dimethyloxaloacetate. Our results also permit the conclusion that if a phosphorylated derivative of OAA is formed as an intermediate in the reaction, the phosphate must be attached to a carboxyl group.

The wheat germ extracts contain a very active malic dehydrogenase as well as the enzymes necessary for the conversion of 3-phosphoglyceric acid (PGA) to PEP. Fumarase is absent, however. It was possible, therefore, to form malate from PGA and carbon dioxide by incubation of the latter two substances with wheat germ extract and reduced diphosphopyridine nucleotide (DPNH), generated with ethanol and alcohol dehydrogenase. Since the OAA is reduced as soon as it is formed, there is little opportunity for non-enzymatic keto-enol tautomerization.

This reaction was carried out in a medium of D_2O, and the malate was isolated as the diphenacyl ester and analyzed for D. In two separate experiments, the malate was found to contain 0.10 and 0.05 atoms of D per molecule. This shows that no enol form of OAA (or a derivative thereof) is a necessary intermediate in the reaction sequence, since, if such were the case, the malate should contain a minimum of one atom of D per molecule. The results also show that malic dehydrogenase causes a direct transfer of hydrogen from DPNH to the carbonyl carbon atom of OAA. The reactions may be formulated by equations 1 through 4:

$$CH_2OPO_3^{=}CHOHCOO^- \rightleftharpoons CH_2OHCHOPO_3^{=}COO^- \rightleftharpoons CH_2{=}COPO_3^{=}COO^- \quad (1)$$

$$CH_2{=}COPO_3^{=}COO^- + CO_2 + OH^- \longrightarrow COO^-CH_2COCOO^- + HPO_4^{=} \quad (2)$$

$$COO^-CH_2COCOO^- + DPNH + H^+ \rightleftharpoons COO^-CH_2CHOHCOO^- + DPN^+ \quad (3)$$

$$CH_3CH_2OH + DPN^+ \rightleftharpoons CH_3CHO + DPNH + H^+ \quad (4)$$

The experiments were carried out as follows: 130 μM. PGA, 160 μM. $MgSO_4$, 200 μM. phosphate buffer of *p*H 7.0, 1 ml. ethanol, 4 μM. DPNH, 1 μg. crystalline yeast alcohol dehydrogenase, 2.0 ml. of dialyzed wheat germ extract[3] and 18 ml. of 95%

(1) R. S. Bandurski and C. M. Greiner, *J. Biol. Chem.* **204**, 781 (1953).

(2) R. Steinberger and F. H. Westheimer, THIS JOURNAL, **73**, 429 (1951).

(3) R. C. Barnett, H. A. Stafford, E. E. Conn and B. Vennesland, *Plant Physiol.*, **28**, 115 (1953).