Polarographic Catalytic Waves of Ultraviolet Irradiated and Urea Denatured Bovine Serum Albumin

In the course of studies on the photochemical effects on albumin solutions we made some interesting observations about changes in the polarographic behavior of albumin (BSA) after irradiation in air-free solutions.

Solutions of 10^{-4} and $10^{-5}M$ BSA at pH 6.7 were exposed to irradiation of an intensity of $8.3 \cdot 10^{-9}$ einstein ml^{-1} sec^{-1} and after various periods of time aliquots of the irradiated solutions were added to an ammoniacal cobaltic buffer to make the BSA concentration $4 \cdot 10^{-7}M$ in all polarographic experiments. From Figure 1 it is seen that the polarogram of native BSA exhibits a double wave (Figure 1A) in more concentrated and a single peak (h_1, Figure 1B) in more dilute solutions. Comparing Figure 1B with 1C it is found that after irradiation the polarogram exhibits a second wave at a potential at which native BSA gives a minimum. The presodium wave (h_3) is not seen after irradiation. The height of wave h_1 decreases upon irradiation, the effect being stronger when the BSA concentration of the irradiated solution is smaller. The height of the second wave also decreases with time of irradiation. The changes are reversed when BSA solutions are irradiated in the presence of air, and the height of the waves increases with time of irradiation. This was found by WENIG and JIROVEC[1] and confirmed in this laboratory.

Amperometric sulfhydryl titrations reveal that disulfide is ruptured upon irradiation in air-free media and the number of broken disulfide groups approaches a limiting value after a certain time of irradiation. Figure 2 illustrates the relation between h_1 and the number of broken disulfide bonds/M BSA. Curve A ($10^{-4}M$ BSA) shows that the fission of the first 6 disulfide groups has little effect on the peak height, but greater changes occur when more of the fissionable disulfide is broken. The situation is similar for a $10^{-5}M$ BSA solution (Figure 2B) in which the number of fissionable disulfide/M BSA is larger than in the more concentrated solution. The polarographic activity is unchanged when all sulfhydryl groups, formed during irradiation, are blocked with iodoacetate. This indicates that sulfhydryl formed during irradiation does not contribute to the polarographic activity of the protein, and structural changes appear to be the determining factor.

Structural changes also occur upon denaturation in urea solutions. Since urea denaturation is partially reversible[2] after dilution, polarograms were first taken with cobaltic buffers which were $4F$ in urea. 2 waves are observed which increase in height with time of denaturation. When the denaturation mixture is added to a cobaltic buffer without urea, denaturation is partially reversed and the height of the first peak (h_1) is almost the same as that of native BSA throughout the entire denaturation period (5 h), while the second wave increases in height with time of denaturation. A denaturation mixture ($4F$ urea, pH 9.2) containing $2M$ of mercuric chloride/M BSA does not give a second wave and the polarogram is identical with that of native BSA throughout the entire period of denaturation (8 h). The same is found with denaturation mixtures at lower pH (6.7 and 5) in the absence of mercury. In urea solutions albumin is unfolded but the number of disulfide groups is not changed during denaturation[3]. At alkaline pH a cross-linking reaction takes place[4]

$$PSH + P\begin{matrix}S\\|\\S\end{matrix} \rightarrow P\text{-}S\text{-}S\text{-}PSH \qquad (1)$$

where P denotes interacting molecules of denatured protein. This reaction is irreversible and its rate decreases with decreasing mercaptide ion concentration which is considerably reduced in the presence of mercury and at low pH. Considering these facts, our experimental results allow the conclusion that the appearance of the second wave in denatured BSA is indicative for cross-linking reaction (1) which progresses continuously during denaturation, while the increase of the height of the first

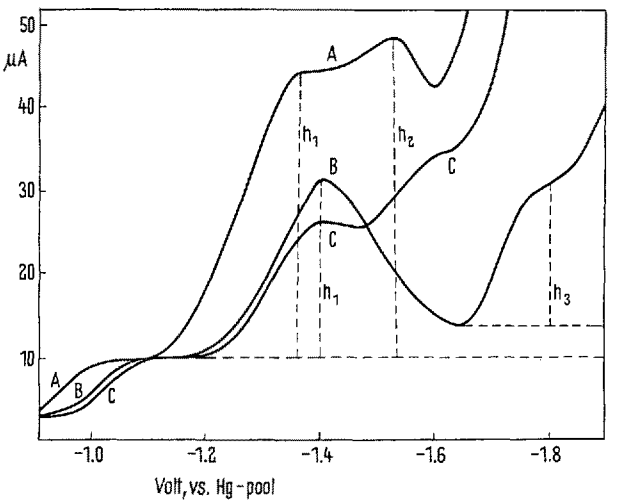


Fig. 1. Polarograms of BSA in ammoniacal cobaltic buffer, (A) $6 \cdot 10^{-6}M$ in native BSA, (B) $4 \cdot 10^{-7}M$ in native BSA, (C) $4 \cdot 10^{-7}M$ in BSA after 5 h irradiation. Irradiated solution: $10^{-4}M$ BSA, 0.025 F Na_2HPO_4-NaH_2PO_4.

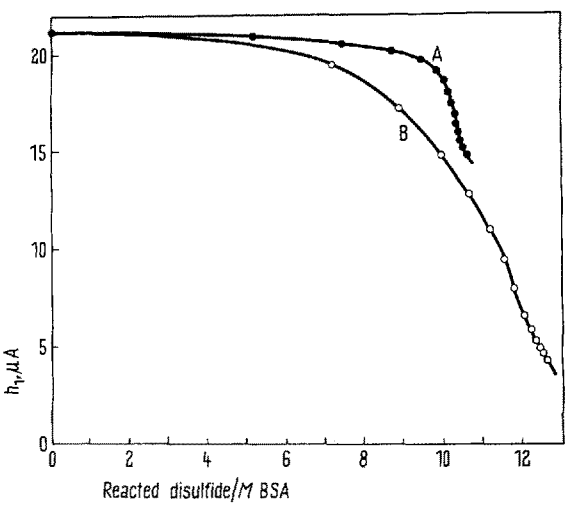


Fig. 2. Height (h_1) of first protein peak vs. number of fissioned (reacted) disulfide groups/M BSA after irradiation of (A) $10^{-4}M$ BSA, (B) $10^{-5}M$ BSA solutions, pH 6.7. Electrolyzed solutions: ammoniacal cobaltic buffer, $4 \cdot 10^{-7}M$ in BSA.

[1] K. WENIG and O. JIROVEC, Biochem. Z. *294*, 405 (1937).
[2] I. M. KOLTHOFF et al., J. Am. chem. Soc. *82*, 4147 (1960).
[3] I. M. KOLTHOFF et al., J. Am. chem. Soc. *79*, 5102 (1957).
[4] W. KAUZMANN, in *The Mechanism of Enzyme Action* (Ed. W. D. McELROY and B. GLASS; The Johns Hopkins Press, Baltimore, Md., USA 1954), p. 70.

wave is characteristic for the fission of hydrogen bonds and unfolding of the protein molecule. Unfolding is reversible and the first wave is therefore unchanged if the urea concentration is reduced after denaturation.

During irradiation in air-free media no unfolding occurs but aggregation gives rise to molecules of high M as reported by RIDEAL and ROBERTS[5] who also found that photooxidative decomposition occurs when albumin is irradiated in the presence of air. Since it is known that larger molecules give smaller catalytic waves, it can be concluded that the smaller waves (h_1) in irradiated air-free BSA solutions are caused by aggregation while the higher waves in the presence of air are the result of photooxidation.

The second wave in irradiated solutions is probably the result of cross-linking reactions between newly formed sulfhydryl and remaining disulfide, although the correctness of this assumption cannot be demonstrated by irradiation experiments with BSA solutions in the presence of mercuric chloride which, if present in sufficient amounts, gives rise to precipitation of protein during irradiation.

The polarographic results obtained with irradiated and urea denatured BSA lead to the conclusion that under proper experimental conditions the height of the first peak is characteristic for the secondary structure and molecular size of the protein, while the appearance of a second wave is indicative for cross-linking reactions.

Guanidine hydrochloride has a specific effect on the catalytic protein waves and can therefore not be used for a polarographic study of denaturation effects[6].

Zusammenfassung. Serumalbumin von Rindern wurde in 2 verschiedenen Konzentrationen mit UV-Licht bestrahlt und die polarographisch-katalytischen Wellen mit denen von Albumin verglichen, das zuvor mit Harnstoff behandelt worden war.

W. STRICKS and J. V. ADVANI

Department of Chemistry, Marquette University, Milwaukee (Wisconsin 53233, USA),
August 22, 1966.

[5] E. K. RIDEAL and R. ROBERTS, Proc. R. Soc., Series A, *205*, 391 (1951).

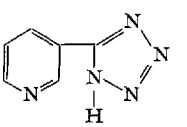
[6] This investigation was supported by the U.S. Army, Medical Research and Development Command, Department of the Army, under Research Contract No. DA-49-193-MD-2146.

5-(3-Pyridyl)tetrazole, a Potent and Long-Acting Lipolysis Inhibitor

The administration of nicotinic acid to man lowers the level of plasma free fatty acids (FFA)[1,2]. The depression of plasma FFA is of short duration and is followed by a rise of plasma FFA above the normal fasting level. Also, nicotinic acid in man, as well as in animals, blocks the catecholamine stimulated mobilization of FFA in vivo, as well as the release of FFA from adipose tissue in vitro. CARLSON and ORÖ[1,3] have suggested that nicotinic acid lowers plasma cholesterol and triglycerides by inhibiting FFA release from adipose tissue.

The rapid metabolic inactivation of nicotinic acid could account for the short duration of plasma FFA lowering. GINOULHIAC et al.[4] have shown that nicotinic acid reaches a peak blood concentration 1 h after the oral administration of 500 mg. This dose of nicotinic acid is cleared from the blood of humans within 4 h. Only 18% of the dose could be recovered unchanged in the urine after 24 h. These observations suggest that an important factor in the removal of nicotinic acid from blood is its conversion to inactive metabolites. The brief sojourn of nicotinic acid in the blood appears adequate to explain the short duration of nicotinic acid-induced FFA depression in man. A lipolysis inhibitor of the same magnitude of activity in vitro as nicotinic acid, but of greater metabolic stability, would be expected to depress plasma FFA for a longer period of time.

5-(3-Pyridyl)tetrazole (I)[5], has some of the important salient structural features of nicotinic acid, i.e. a pyridine nucleus with an acidic function at the 3 position. HERBST and WILSON[6] have shown that 5-substituted tetrazoles are acidic. More importantly, the tetrazole function is metabolically stable. For example, about 75% of a dose of pentylenetetrazole (Metrazol) can be isolated from rat urine unchanged[7]. I was prepared by an improved procedure for the synthesis of 5-substituted tetrazoles[8]. As expected, the apparent ionization constant of I ($pK_a = 4.1$) was quite similar to that of nicotinic acid ($pK_a = 4.5$)[9]. 5-(3-Pyridyl)tetrazole showed lipolysis inhibitory activity which was similar in some respects to nicotinic acid, but differed significantly and interestingly in other aspects.



I

[1] L. A. CARLSON and L. ORÖ, Acta med. scand. *172*, 641 (1962).
[2] L. A. CARLSON and L. ORÖ, J. Atheroscler. Res. *5*, 436 (1965).
[3] L. A. CARLSON, Acta med. scand. *173*, 719 (1963).
[4] E. GINOULHIAC, L. T. TENCONI and F. M. CHIANCONE, Nature *193*, 948 (1962).
[5] W. J. VAN DER BURG, Recl Trav. chim. Pays-Bas Belg. *74*, 257 (1955).
[6] R. M. HERBST and K. R. WILSON, J. org. Chem. *22*, 1142 (1947). For example, the apparent ionization constant of 5-phenyltetrazole (pK_a 4.5) is slightly greater than that of the corresponding carboxylic acid, benzoic acid (pK_a 5.1).
[7] D. W. ESPLIN and D. M. WOODBURY, J. Pharmac. exp. Ther. *118*, 129 (1956).
[8] W. G. FINNEGAN, R. A. HENRY and R. LOFQUIST, J. Am. chem. Soc. *80*, 3908 (1958).
[9] The apparent ionization constants were determined by Mr. THOMAS J. TOOLAN by potentiometric titrations, using a Beckman Model G pH meter, in ethanol-H_2O (50% v/v) medium with standard 0.5 N sodium hydroxide. The apparent pK_a values correspond to the pH at the 50% neutralization point in these titration curves.