and extracted with ether (3 × 100 ml). The combined ether extracts were washed with saturated sodium chloride solution, dried over sodium sulphate and the solvent evaporated under reduced pressure. The yield was 8.2 g of a brown liquid, which was purified by chromatography on neutral alumina (aluminium oxide Woelm activity grade I 80 g, 1.6 cm diam.). The ester was eluted with ether. The eluent was evaporated *in vacuo* to give 7.5 g of a slightly brown liquid. Distillation under reduced pressure afforded 6.8 g of the desired ester in 82 % purity. (GLC: 15 % Carbowax 1500 on Celite 545 60–100 mesh). B.p. 71–73°/11 mm. The ester was used in the subsequent step without further purification.

trans-*3-Methyl-2-hexenoic acid.* 1.60 g of the impure ester was dissolved in a mixture of 20 ml 1 M sulphuric acid and 25 ml of acetic acid and heated to reflux for 5 h. The solvent was evaporated *in vacuo* to approx. 5 ml, made alkaline with 2 M sodium hydroxide and extracted with ether (2 × 20 ml). The aqueous layer was acidified with 2 M hydrochloric acid and extracted with ether (3 × 50 ml). The combined ether extracts were washed with saturated sodium chloride solution, dried over sodium sulphate and the solvent evaporated *in vacuo* affording 0.67 g of the acid which crystallized on cooling. Recrystallization from ethanol-water gave 0.35 g of the desired acid as white crystals. M.p. 37–38°. The purity was judged to be better than 99 % by GLC, (10 % diethylene glycol adipate and 2 % phosphoric acid 85 % on acid-washed Chromosorb W 60–80 mesh). (Found: C 66.05; H 9.41; O 24.62. Calc. for $C_7H_{12}O_2$: C 65.60; H 9.44; O 24.96.)

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1. Smith, K., Thompson, G. F. and Koster, H. D. *Science* **166** (1969) 398.
2. Canonica, L., Fedeli, E. and Castelnuovo, A. *Gazz. Chim. Ital.* **87** (1957) 998.
3. Corey, E. J. and Katzenellenbogen, J. A. *J. Am. Chem. Soc.* **91** (1969) 1851.
4. Gilman, H. and Gaj, B. J. *J. Org. Chem.* **22** (1957) 1165.
5. *Org. Syn. Coll. Vol. IV* insertion page between pp. 792–793.

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Influence of Hydrophobic Interactions and Hydration on the Radiosensitivity of a Protein

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The radiosensitivities of organisms as well as of some macromolecules *in vitro* have been shown to be strongly influenced by the amount of hydration water present.[1-6] Generally, such systems are less sensitive in a moderately hydrated than in a desiccated form. The mechanism for this protection has been a matter of speculation only.

Another class of compounds, which has been discussed in connection with the radiosensitivities of biological macromolecules, are those which have low-lying electronic excited states. It has been suggested that such groups if present within or in the vicinity of a macromolecule might protect this by acting as "sinks" for excitation energy.[4,7-9] Such protection has been demonstrated with proteins exposed to ultraviolet light.[10,11]

In this work, the influence of hydration water and other adsorbed compounds on the radiosensitivity of bovine serum albumin has been studied in order to throw some light on these problems. Radiation injury has been assayed as the loss of solubility.

Materials and methods. Bovine serum albumin (BSA) was a crystalline preparation from Armour and Co. Results obtained with this material as such or after dissolution in distilled water and lyophilization did not deviate appreciably. DNS-BSA, the conjugate with 1-dimethylaminonaphthalene-5-sulfonate, was prepared as described in an earlier paper.[12] It had a molar ratio dye/protein of 2.7–3.0. The solid form was obtained by lyophilization.

BSA has five strong binding sites for 1-anilino-8-naphthalenesulfonate (ANS). Solid ANS-BSA was obtained by lyophilization of a concentrated solution in distilled water of the components in this molar ratio. BSA with

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a 10:1 excess of caprylic acid was prepared in a similar way.

100 mg samples of the protein or protein conjugate were incubated for three days over various solutions to obtain specified water contents [12] and then irradiated in controlled atmospheres in a ^{60}Co-source. After irradiation the samples were all exposed to the same atmosphere in one closed vessel for about 24 h. They were then dissolved in 2 ml 0.1 M neutral phosphate buffer. The insoluble residues were separated from the solutions by centrifugation at about 10 000 *g*. The residues were repeatedly washed with distilled water and recentrifuged. The remaining pellets were taken to dryness at 105°C overnight in the previously weighed centrifuge tubes, and the dry weights determined from the differences in weights.

The yield of insoluble residue in irradiated BSA is dependent on, *e.g.*, pH and ionic strength of the solvent.[13] The standardized conditions selected for this study, however, yield reproducible results and seem to generate optimal information.

Results and discussion. Fig. 1 shows the extents of insolubility produced at various water contents at three different dose levels. The experiments were confined to doses which yield relatively low degrees of insolubilization because the accuracy of the assay decreases drastically at higher doses, probably due to the occlusion of soluble protein by the precipitates. If

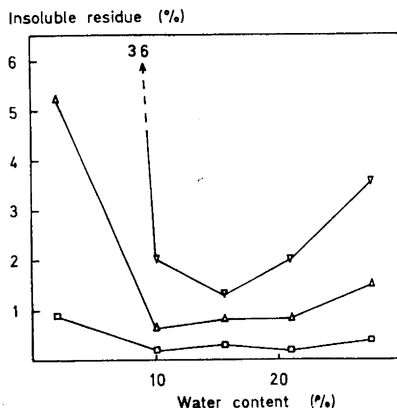

Fig. 1. Production of insoluble residue in BSA irradiated at various water contents. □, 5 Mrads; △, 10 Mrads; ▽, 15 Mrads. Insoluble residues of the corresponding unirradiated samples (0.1 %) have been subtracted from the presented values.

plotted *versus* dose in a log/log diagram, the values corresponding to each water content form practically straight lines, and from such diagrams (not reproduced here), the doses corresponding to various degrees of insolubilization are obtained by interpolation. The doses corresponding to 1 and 2 % insolubilization are plotted in Fig. 2. It is seen that the water content

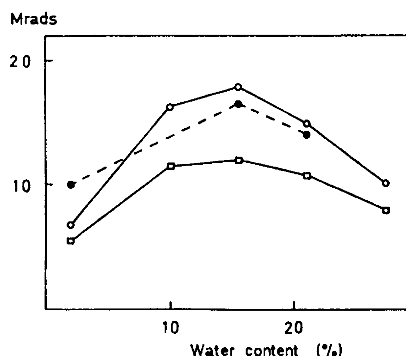

Fig. 2. Doses required to produce insolubilization. Squares signify insolubilization of 1 % and circles of 2 % of the material. Open circles are for BSA and closed for DNS-BSA.

influences the radiosensitivity expressed in this way by as much as a factor of 2.5. Experiments with DNS-BSA showed that the conjugation with DNS results in a reduction of the sensitivity at low water content (Fig. 2). The differences in sensitivities at higher water contents seen in the graph are probably not significant.

BSA was also irradiated with one molecular species similar to DNS and one which also binds relatively strongly to the protein, but which lacks the aromatic character and the spectral properties of the dyes. Samples of BSA, DNS-BSA, ANS-BSA, and caprylate-BSA were dried to a water content of 2 % and then irradiated with doses large enough to cause appreciable insolubilization. The results are summarized in Table 1.

The data obtained in this investigation show that in the order of 5 – 10 hits are required to produce an insoluble molecule. This is in agreement with the results obtained by Alexander and Hamilton.[13] The water content dependence found here deviates from that found in an experiment reported by Alexander and Hamilton,

Table 1. Radiation-induced insoluble residue from BSA conjugates with 2 % hydration water expressed in percent of the corresponding values for pure BSA.

Conjugate	10 Mrads	15 Mrads
DNS-BSA	15	37
ANS-BSA	28	—
Caprylate-BSA	24	42

but since their experiment was run in nitrogen, no direct comparison can be made.

In a separate study [14] it was shown that hydration water influences neither dimerization of BSA nor structural changes in this protein produced by the absorption of radiation energy corresponding to one or two hits per molecule. It thus seems that at least in this protein hydration water influences only gross changes, which render the protein insoluble. The early explanation to the protective effect of hydration water, *viz.* a strong influence on the transfer of electronic excitation energy [3,4] seems unlikely in view of later measurements of transfer efficiencies.[12] The most likely explanation is that hydration water increases the stability of the protein structure.

The reductions of the radiosensitivity caused by DNS, ANS, and caprylic acid were found to be of similar magnitudes (Table 1). These compounds have in common the property to interact with hydrophobic regions in BSA.[15–17] It may thus be concluded that at least an important part of the protection is due to hydrophobic interactions with the protein. Fatty acids have been shown to protect BSA against denaturation,[18,19] and heme has been shown to stabilize heme proteins.[11] The fact that ANS was not found to stabilize the apoenzyme of peroxidase against heat denaturation [11] does not exclude a protection in the case of BSA. The association constant — and thus the interaction energy — is much higher for this protein than for apoperoxidase.[16,20]

Recent experimental studies of excitation transfer in ANS-protein complexes [20,21] have shown that the transfer of electronic excitation energy is efficient only in cases of large spectral overlap. Consequently, it does not seem likely that groups of this type would act as a sort of general sink for excitation energy.

It appears from this study that the radiosensitivity of a protein may be reduced by the adsorption of water and of compounds binding to hydrophobic regions. The mechanism for the protection is probably in both cases a stabilization of the protein structure.

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1. Ehrenberg, L. *Svensk Kem. Tidskr.* **67** (1955) 207.
2. Kaplan, R. W. *Naturwiss.* **42** (1955) 184.
3. Kaplan, R. W. *Naturwiss.* **42** (1955) 466.
4. Ehrenberg, L., Jaarma, M. and Zimmer, E. C. *Acta Chem. Scand.* **11** (1957) 950.
5. Metzger, K. Z. *Z. allgem. Mikrobiol.* **1** (1960) 29.
6. Augustinsson, K.-B., Jonsson, G. and Sparrman, B. *Acta Chem. Scand.* **15** (1961) 11.
7. Reid, C. *Excited States in Chemistry and Biology*, Butterworths, London 1957, p. 184.
8. McLaren, A. D. and Shugar, D. *Photochemistry of Proteins and Nucleic Acids*, Pergamon, Oxford 1964, p. 112.
9. Dainton, F. S. In Phillips, G. O. *Energy Transfer in Radiation Processes*, Elsevier, Amsterdam 1966, p. 1.
10. Slayer, H. S. and Hall, C. E. *J. Mol. Biol.* **8** (1964) 593.
11. Rosén, C.-G. and Nilsson, R. *Biochim. Biophys. Acta* **236** (1971) 1.
12. Rosén, C.-G. and Ahnström, G. *Int. J. Radiat. Biol.* **9** (1965) 435.
13. Alexander, P. and Hamilton, L. D. G. *Radiat. Res.* **15** (1961) 193.
14. Rosén, C.-G. *Int. J. Radiat. Biol. In print.*
15. Teresi, J. D. and Luck, J. M. *J. Biol. Chem.* **194** (1952) 823.
16. Weber, G. and Young, L. B. *J. Biol. Chem.* **239** (1964) 1415.
17. Chen, R. F. *Arch. Biochem. Biophys.* **128** (1968) 163.
18. Boyer, P. D., Ballou, G. A., Luck, J. M. and Rice, R. G. *J. Biol. Chem.* **162** (1946) 181.
19. Boyer, P. D., Ballou, G. A. and Luck, J. M. *J. Biol. Chem.* **162** (1946) 199.
20. Rosén, C.-G. *FEBS Letters* **6** (1970) 158.
21. Stryer, L. *J. Mol. Biol.* **13** (1965) 482.

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