Table II. Effect of dietary fats on liver enzymes

Enzyme	Diets				
	Safflower oil	Coconut oil	Hydrogenated vegetable fat	Fat-free	Fat-free with cholesterol
α-glycerol *p*-dehydrogenase (E.C.1.1.1.8)	8.9±1.5	10.1±2.1	9.2±1.9	13.4±2.5	14.5±2.7
Glycerol kinase (E.C.2.7.1.30)	0.85±0.15	0.9±0.15	0.9±0.19	1.2±0.1	1.2±0.12
Citrate cleavage enzyme (E.C.4.1.3.7)	2.25±0.20	5.7±0.20	5.3±0.35	8.5±1.2	10.1±1.2
Malic enzyme (E.C.1.1.1.38)	3.4±1.2	10.75±1.6	10.55±2.25	17.6±4.56	18.9±4.5
Malate dehydrogenase (E.C.1.1.1.37)	25.5±4.7	27.9±4.7	27.0±5.2	28.5±3.8	28.5±4.5
Isocitrate dehydrogenase (E.C.1.1.1.42)	24.5±3.8	25.7±3.7	26.5±2.9	28.9±4.9	29.5±4.7

The above values are units of the enzyme/g liver. 1 unit of the enzyme in all the cases is taken as the amount which causes an optical density change of 0.001 per min in the system. The figures are the mean of 8 values along with SD.

provide supporting evidence for the hypothesis that EFA deficiency causes alterations in certain structures of the animal tissues[12]. Previous work from this laboratory (unpublished data) revealed that high amounts of dietary saturated fat caused a marked decrease in PUFA levels in the mitochondria, especially in the mitochondrial phospholipids, and the structural alterations were probably caused by the lack of PUFA in the tissue. These in turn might be responsible for the differences noted in the mitochondrial enzyme levels.

The general nature of the enzymatic changes noted here are consistent with the previous reports which indicated that as the fat content of the diet decreased, rates of fatty acid synthesis and the enzyme activities involved in these functions increased. In addition, our results also show that the activities of many of these enzymes are altered as a function of unsaturated fat intake. The differences in the liver fatty acid profile due to the feeding of different fats to rats, when accompanied by the changes in the enzymatic levels noted here, are in support of the observations made by ALLMAN et al.[3].

[1] S. ABRAHAM, L. KOPELOVICH and I. L. CHAIKOFF, Biochim. biophys. Acta *93*, 185 (1964).

[2] R. L. BALDWIN and M. RONNING, J. Dairy Sci. *49*, 688 (1966).

[3] D. W. ALLMAN, D. W. HUBBARD and D. M. GIBSON, J. Lipid Res. *6*, 63 (1965).

[4] W. BORTZ, S. ABRAHAM and I. L. CHAIKOFF, J. biol. Chem. *238*, 1266 (1963).

[5] R. L. BALDWIN, M. RONNING, C. RADANOVICS and G. PLANGE, J. Nutr. *90*, 47 (1966).

[6] R. L. BALDWIN and P. MILLIGAN, J. biol. Chem. *241*, 2058 (1966).

[7] M. S. KORNACKER and J. M. LÖWENSTEIN, Biochim. biophys. Acta *84*, 490 (1964).

[8] J. W. YOUNG, E. SHRAGO and H. A. LARDY, Biochemistry *3*, 1687 (1964).

[9] S. V. PANDE, S. V. PARVINKHAN and T. A. VENKATASUBRAMANIAM, Biochim. biophys. Acta *84*, 239 (1964).

[10] E. M. WISE JR. and E. E. BALL, Proc. natn. Acad. Sci., USA *52*, 1255 (1964).

[11] T. HAYASHIDA and O. W. PORTMANN, Proc. Soc. exp Biol. Med. *103*, 656 (1960).

[12] A. L. WALKER and F. A. KUMMEROW, J. Nutr. *82*, 329 (1964).

ESR-Spectroscopic Changes on Enzymatic Depolymerization of Spin-Labelled Amylose

R. DARCY and K. F. MCGEENEY

Department of Chemistry, University College, Belfield, Dublin 4 (Ireland); and Department of Medicine and Therapeutics, University College, Dublin 4 (Ireland), 5 April 1976.

Summary. The ESR-spectra of nitroxyl-labelled amyloses (molecular weight 14,000) show anisotropic effects. Depolymerization with α-amylase alters the spectra, and the rate of change is dependent on concentration of enzyme.

When a nitroxyl group is introduced into a macromolecule, the ESR spectrum of the spin-labelled compound in solution reflects its molecular size. The relatively slow tumbling of the large molecule causes imperfect averaging of anisotropic magnetic interactions, and the resulting spectrum differs from that observed for a small nitroxide. The affect has been applied in the study of proteins[1] and synthetic polymers[2]. The expectation that such effects would be observed also for spin-labelled amylose formed the basis of the present work[3]. Since the ESR-spectrum would depend on molecular size, it should furthermore be sensitive to depolymerization. The action of amylase could therefore be recorded as a continuously changing spectrum. Synthetic dyes have been linked to amylose substrates for the purpose of assaying amylase[4–6]. These methods, however, all involve sampling, whereas the use of a spin-labelled substrate offers the prospect of

[1] C. L. HAMILTON and H. M. MCCONNELL, in *Structural Chemistry and Molecular Biology* (Eds. A. RICH and N. DAVIDSON; W. H. Freeman and Co., San Francisco 1968), p. 115.

[2] A. T. BULLOCK, G. G. CAMERON and P. M. SMITH, J. phys. Chem. *77*, 1635 (1973).

[3] Preliminary communications entitled *Application of ESR to the Study of α-Amylase Activity* made to the First European Clinical Chemistry Congress, München, W. Germany, April 1974; and to the Annual Congress of the Chemical Society, York, Britain, April 1975. The ESR-spectra of spin-labelled derivatives of cellulose have been reported by D. GAGNAIRE and L. ODIER, Bull. Soc. chim. France (*1974*), 2325.

[4] J. A. THOMA, J. E. SPRADLIN and S. DYGERT, in *The Enzymes* (Ed. BOYER; Academic Press, New York and London 1971), vol. 5, p. 115.

[5] H. RINDERKNECHT, P. WILDING and B. J. HAVERBACK, Experientia *23*, 805 (1967).

[6] M. CESKA, E. HULTMAN and B. G.-A. INGELMAN, Experientia *25*, 555 (1969).

observing fast depolymerization continuously. A nitronyl nitroxide **1** and a piperidine nitroxide **3** were used as labelling reagents in the present study.

Materials and methods. 2-Chloromethyl-1,3-dioxy-4, 4,5,5-tetramethyl-4,5-dihydroimidazole **1** and the methoxymethyl compound **2** were prepared by general methods[7] described for nitronyl nitroxides. 4-Isothiocyanato-2, 2, 6, 6-tetramethylpiperidinooxyl **3** was supplied by Syva (Palo Alto, California). The amylose (Merck soluble, dry according to Zulkowsky) was treated with the chloromethyl-label **1** in dry dimethylsulphoxide with silver oxide as catalyst for 4 days at room temperature. The catalyst was removed by filtration and gradual addition of methanol to the filtrate. The labelled amylose was finally precipitated by saturating the solution with methanol. Treatment with the isothiocynato-label **3** was carried out in dimethylsulphoxide for 3 days at room temperature and 2 h at 40 °C. ESR-spectra were recorded on a Decca X-1 spectrometer with manganese-magnesium oxide as calibration standard. Modulation amplitudes used were less than half the line-widths. The concentration of spin-labelled amylose was 30 mg/ml H_2O.

Results and discussion. Amylose alkylated with 2-chloromethyl-1,3-dioxy-4,4,5,5-tetramethyl-4,5-dihydro-imidazole **1** showed line broadening in the ESR-spectrum (Figure 1). The shape of the spectrum was unchanged at lower concentrations and also when unlabelled amylose was added to double the total carbohydrate concentration. Therefore, although broadened spectra may be obtained for nitronyl nitroxides in viscous solvents[8], the effect here is due to attachment of the nitroxyl system to the macromolecule. This is also evident from comparison with the spectrum (Figure 2) of the model compound **2**. This compound is structurally similar to the labelled amylose in the immediate vicinity of the nitroxyl group, as confirmed by UV-spectroscopy (310, 320 nm), but has a much lower molecular weight. Both spectra have the multiplicity of 5×3 lines due to hyperfine coupling by 2 nitrogen and 2 hydrogen nuclei[7], but that of the amylose is less resolved due to line-broadening. The ESR-spectrum of similarly labelled maltose also shows greater resolution than that of the amylose.

Reaction of the labelled amylose with α-amylase resulted in a spectrum of increased resolution (Figure 3). Graphs of line height against time for various enzyme concentrations took the form shown (Figure 4). These show regular dependence on enzyme concentration, and drop-off in rate with inhibition by product. The amylose had an approximately 40% lower rate of enzymolysis than normal amylose, which compares well with other artificial, for example, dyed[4–5], substrates used to determine enzyme activity.

The ESR-spectrum of amylose treated with 4-isothiocyanato-2,2,6,6-tetramethylpiperidinooxyl **3** consisted of 3 lines of different intensities. In contrast, 3 almost identical lines are observed in the spectrum of the free nitroxide label[9]. Treatment of the labelled amylose with α-amylase produced increases in the line-intensities (Figure 5) tending towards equalization. Dependence of these changes on enzyme concentration was similar to that for the starch labelled with the nitronyl nitroxide **1**. Motion of the label relative to the macromolecule is preferably minimal, and nitronyl nitroxide derivatives are known to exhibit hindered rotation of the ring[7]. The nitronyl nitroxide label does not however seem to have a greater sensitivity to depolymerization than the piperidine nitroxide. Also, the more complex spectrum of the former system increases the difficulty of interpreting anisotropic effects[8].

Interpretation in kinetic terms of the spectroscopic changes observed on enzymolysis requires information on the relationship between molecular weight and spectrum. Rotational correlation times (τ_c), which are a measure of molecular motion and hence of molecular size, may be calculated for spin-labelled macromolecules as a function of line-shapes[10]. The spectrum of the amylose labelled

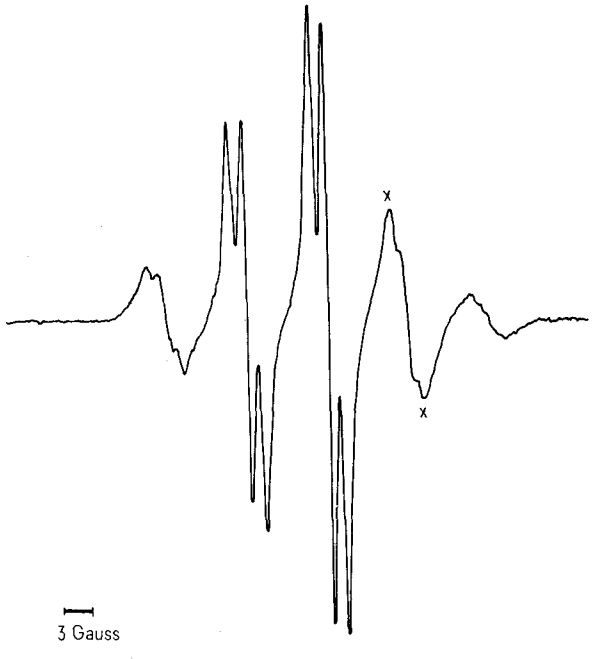


Fig. 1. ESR-spectrum of 1-labelled amylose.

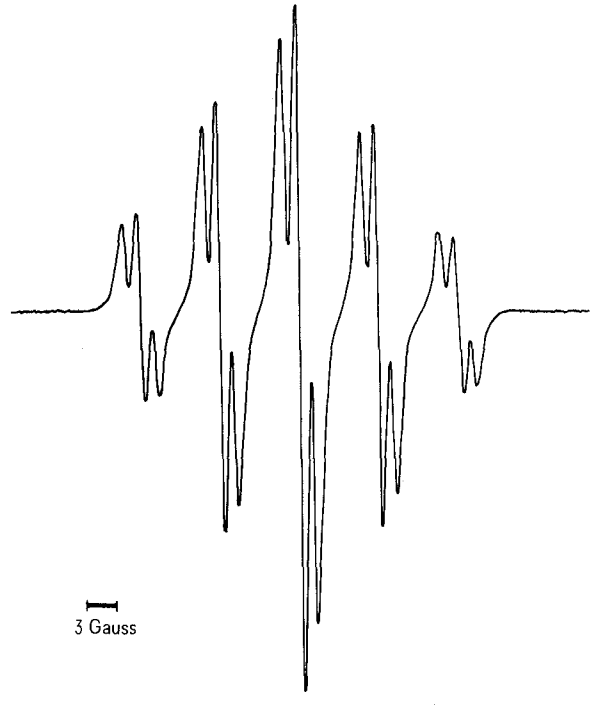
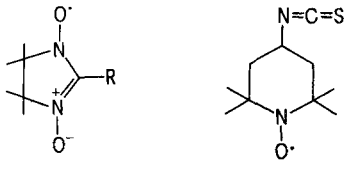


Fig. 2. ESR-spectrum of 2.



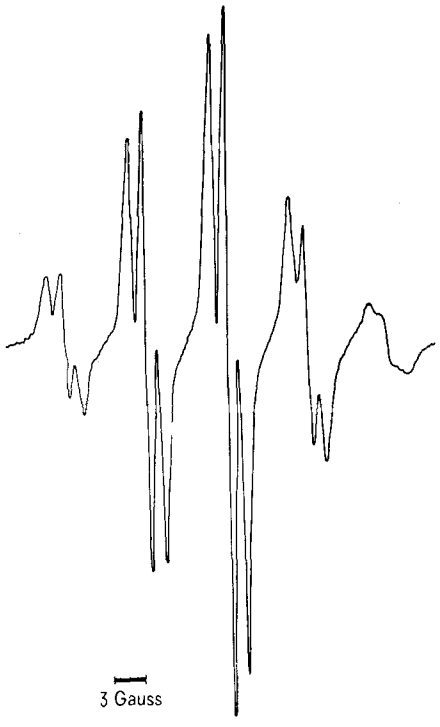


Fig. 3. ESR-spectrum of 1-labelled amylose after treatment with α-amylase.

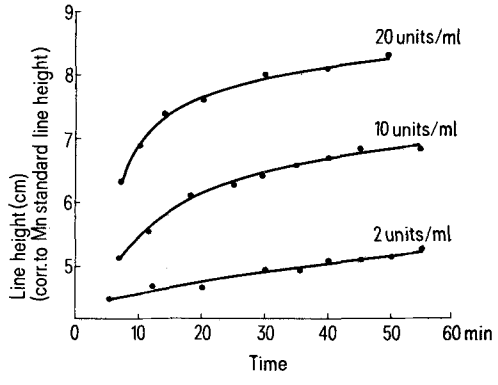


Fig. 4. Plot of line height (× × in Figure 1) of ESR-spectrum of 1-labelled amylose against time for various concentrations of α-amylase.

with reagent **3** yields the value $\tau_c = 1.1 \times 10^{-9}$ sec. The value of τ_c will reflect molecular size for spherically symmetric motion of a rigid molecule. The conformation of amylose in aqueous solution has been a matter of controversy until relatively recently when, on the basis of viscosity measurements, the random coil conformation was assigned[11]. This is more consistent with a spherical shape than the helical conformation, which would result in an extended, rod-like molecule. The strong dependence of the ESR-spectrum on initial rate of enzymolysis indicates that motion of the entire molecule, rather than of chain segments or of the label relative to the chain, is important in deciding the shape of the spectrum. This is consistent with the observation for polystyrene[2] that reorientation of the chain becomes important spectroscopically only at

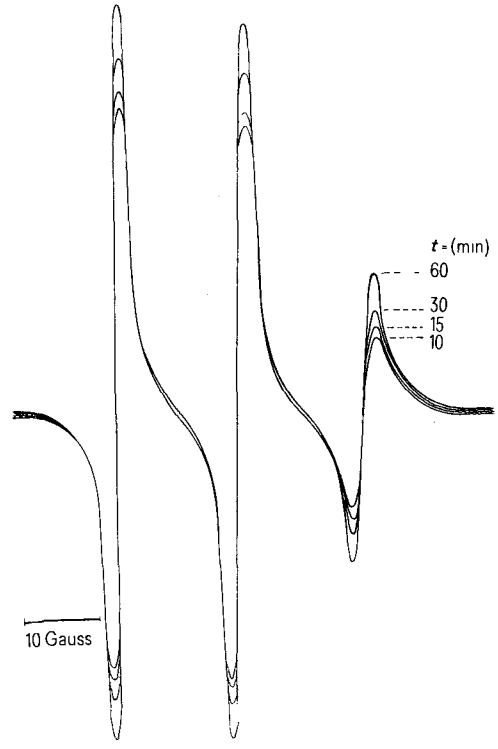


Fig. 5. Changes in ESR-spectrum of 3-labelled amylose during treatment with α-amylase.

molecular weights above 50,000 (500 monomer units). This may be compared with the molecular weight of 14,000 (100 glucose units) for the amylose used in the present study. Segmental reorientation should in any case be less important for amylose, in which there is the possibility of intramolecular hydrogen-bonding. Interpretation of the spectra in kinetic terms is being developed.

The technique shows some promise as a continous spectroscopic method of observing depolymerization reactions, and may find application in particular to fast reactions or the detection of subtle kinetic differences between mutant enzymes. It should also be noted that the reverse spectroscopic changes would take place on polymerization of labelled reactant, so that the method may be applicable to certain polymerization processes.

[7] E. F. Ullman, J. H. Osiecki, D. G. B. Boocock and R. Darcy, J. Am. chem. Soc. *94*, 7049 (1972).
[8] J. A. D'Anna and J. H. Wharton, J. chem. Phys. *53*, 4047 (1970).
[9] See, for example, R. Brière, H. Lemaire and A. Rassat, Bull. Soc. chim. France (*1965*), 3273.
[10] A. S. Waggoner, O. H. Griffith and C. R. Christensen, Proc. natn. Acad. Sci. USA *57*, 1198 (1967).
[11] W. Banks and C. T. Greenwood, Carbohydrate Res. *21*, 229 (1972).