than 50%. However, no appreciable variation of the α-helix CD pattern is observed.

This initial rapid step is followed by a further slow variation of CD spectra over a much longer period of time (about 3 days). This last slow step is characterized by the gradual appearance of an intense side-chain CD couplet, whose chirality is opposite with respect to that observed in pure TMP (Figure 1). At the same time the spectra become more and more distorted in the peptide region, with red shifting of the 222-nm band toward 225 nm and progressive flattening of the 208- and 193-nm bands (Figure 2).

CD spectra exhibit the same distortions as α-helical poly(L-glutamic acid) does, when aggregation progresses below pH 4.[18-21] The same features of the spectra are observed when CD measurements are carried out on turbid suspensions of membrane proteins.[22,23] Indeed, this type of spectra has been used as a diagnostic tool to detect aggregates of α-helices,[24] as the main sources of these distortions are due to the absorption flattening effect and to the differential scattering of left and right circularly polarized light, produced by ordered aggregates of chromophores.[21-27]

Therefore, the initial step brought about by the addition of the polar solvent must be related to a change of the conformation of the side chains involved in a regular array on the periphery of the helix backbone.[17] In contrast, the subsequent slow step should be associated with an aggregation process of the helical polypeptide chains.[20-32] Accordingly, light-scattering intensity increases with time and long aging of the solutions.[33]

Irradiation at λ = 370 nm completely cancels the side-chain CD bands. At the same time in the peptide region, CD spectra revert to the initial not distorted ones (Figure 2). As can be observed in the CD spectra recorded on the freshly prepared solutions, the photoisomerization itself of the azo chromophore does not affect the CD spectra in the peptide region. So the light effect cannot be masked by eventual differential contributions of the trans and the cis configuration of azo chromophores. The variation of the spectra upon illumination can be well interpreted as resulting from dissociation of the aggregates induced by light.[34]

By dark adaptation the side-chain CD bands gradually appear again and the spectra revert again to the distorted ones with approximately the same time scale, thus confirming the reversibility of the change. The cycle can be repeated at any time of the aging of the solutions (Figure 2).

Aggregation may be formed between azobenzene moieties in the presence of water, through hydrophobic interactions and ordered stacking of azo groups.[28-32,35,36] These interactions are favored in dark-adapted samples, as *trans*-azobenzene moieties are planar and very hydrophobic. Light induces the disaggregation process, as the cis form is more polar and not planar,[37] thus enhancing the polymer solubility and inhibiting the associative conditions. Therefore, it is likely that the different polarity and the different geometry between the trans and the cis form of the azo moieties provide the driving force for the photoinduced aggregation–disaggregation process.[38]

In naturally occurring photoreceptors, photoexcitation of photochromic molecules is known to induce reversible changes both in the conformation[39,40] and in the aggregation[41,42] of the attached protein matrix. Thus, the described data can be particularly relevant to the phenomenon of photoregulation in biological systems.

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(18) Long, M. M.; Urry, D. W. In "Membrane Spectroscopy"; Grell, E., Ed.; Springer-Verlag: Berlin, 1981; pp 143–171.
(19) Urry, D. W.; Long, M. M. In "Methods in Membrane Biology"; Korn, E. D., Ed.; Plenum Press: New York, 1974; Vol. 1, pp 105–141.
(20) Urry, D. W.; Hinners, T. A.; Masotti, L. *Arch. Biochem. Biophys.* **1970**, *137*, 214–221.
(21) Gordon, D. J. *Biochemistry* **1972**, *11*, 413–420.
(22) Rosenheck, K.; Schneider, A. S. *Proc. Natl. Acad. Sci. U.S.A.* **1973**, *70*, 3458–3462.
(23) Gitter-Amir, A.; Rosenheck, K.; Schneider, A. S. *Biochemistry* **1976**, *15*, 3131–3137.
(24) Maeda, H.; Hato, H.; Ikeda, S. *Biopolymers* **1984**, *23*, 1333–1346.
(25) Bayley, P. In "An Introduction to Spectroscopy for Biochemists"; Brown, S. B., Ed.; Academic Press: London, 1980; pp 148–234.
(26) Brith-Lindner, M.; Rosenheck, K. *FEBS Lett.* **1977**, *76*, 41–44.
(27) Philipson, K. D.; Sauer, K. *Biochemistry* **1973**, *12*, 3454–3458.
(28) Rinnert, H.; Thirion, C.; Dupont, G.; Lamatre, J. *Biopolymers* **1977**, *16*, 2419–2427.
(29) Smerdon, J. J.; Isenberg, I. *Biochem. Biophys. Res. Commun.* **1973**, *55*, 1029–1034.
(30) D'Anna, J. A., Jr.; Isenberg, I. *Biochemistry* **1974**, *13*, 4992–4997.
(31) Sato, S.; Ebert, C. D.; Kim, S. W. *J. Pharm. Sci.* **1983**, *72*, 228–232.
(32) Rueger, M.; Bienert, M.; Mehlis, B.; Gast, K.; Zirwer, D.; Behlke, J. *Biopolymers* **1984**, *23*, 747–758.
(33) Attempts to follow aggregation processes by means of viscosity measurements were unsuccessful. When concentration were sufficiently high, in fact, polymer precipitated soon after addition of water to the TMP solution.
(34) The trans-to-cis isomerization always cancels the side-chain CD bands at any conditions, so their disappearance upon irradiation cannot be considered a probe for the dissociation.
(35) Tazuke, S.; Iwaya, Y.; Hayashi, R. *Photochem. Photobiol.* **1982**, *35*, 621–626.
(36) Horne, D. S. *Biopolymers* **1984**, *23*, 989–993.
(37) In *cis*-azobenzene in crystalline state, the planes of the two phenyl rings form a skew angle of 56° (Hampson, C. C.; Monteath Robertson, J. *J. Chem. Soc.* **1941**, 409–413).
(38) In principle aggregation could occur also through hydrogen-bonding between the unmodified COOH side chains. However, aggregation in the dark does not occur in the presence of about 5 mol % low molecular weight azobenzene, which inhibits association of macromolecules by intercalating with the azo side chains.
(39) Erlanger, B. F. *Annu. Rev. Biochem.* **1976**, *45*, 267–283.
(40) Borochov-Neori, H.; Montal, M. *Biochemistry* **1983**, *22*, 197–205.
(41) McCaslin, D. R.; Tanford, C. *Biochemistry* **1981**, *20*, 5212–5221.
(42) Borochov-Neori, H.; George Fortes, P. A.; Montal, M. *Biochemistry* **1983**, *22*, 206–313.

Dielectric Relaxation Studies Demonstrate a Peptide Librational Mode in the Polypentapeptide of Elastin

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The elastic fiber of biological tissues contains the repeating pentameric sequence $(\text{L-Val}^1\text{-L-Pro}^2\text{-Gly}^3\text{-L-Val}^4\text{-Gly}^5)_n$ where *n* is as much as 13 in chick tissue without a single variation.[1,2] This polypentapeptide of elastin has been synthesized with *n* greater than 100;[3] it has been variously cross-linked and found to be elastomeric.[3-5] Conformational studies[6-10] have led to the description of a class of helical conformations called β-spirals[11]

(1) Sandberg, L. B.; Soskel, N. T.; Leslie, J. B. *N. Engl. J. Med.* **1981**, *304*, 566.
(2) Sandberg, L. B., private communication.
(3) Urry, D. W.; Prasad, K. U. In "Biocompatibility of Natural Tissues and Their Synthetic Analogues; Williams, K. F., Ed.; CRC Press, Inc.: Boca Raton, FL, in press.
(4) Urry, D. W.; Okamoto, K.; Harris, R. D.; Hendrix, C. F.; Long, M. M. *Biochemistry* **1976**, *15*, 4083.
(5) Urry, D. W.; Wood, S. A.; Harris, R. D.; Prasad, K. J. In "Polymers as Biomaterials" Shalaby, S. W., Horbett, T., Hoffman, A. S., Ratner, B., Eds.; Plenum Publishing Corp.: New York, in press.
(6) Urry, D. W.; Long, M. M. *CRC Crit. Rev. Biochem.* **1976**, *4*, 1.
(7) Urry, D. W.; Trapane, T. L.; Sugano, H.; Prasad, K. U. *J. Am. Chem. Soc.* **1981**, *103*, 2080.
(8) Venkatachalam, C. M.; Khaled, M. A.; Sugano, H.; Urry, D. W. *J. Am. Chem. Soc.* **1981**, *103*, 2372.
(9) Cook. W. J.; Einspahr, H. M.; Trapane, T. L.; Urry, D. W.; Bugg, C. E. *J. Am. Chem. Soc.* **1980**, *102*, 5502.
(10) Venkatachalam, C. M.; Urry, D. W. *Macromolecules* **1981**, *14*, 1225.
(11) Urry, D. W. *Adv. Exp. Med. Biol.* **1974**, *43*, 211–243.

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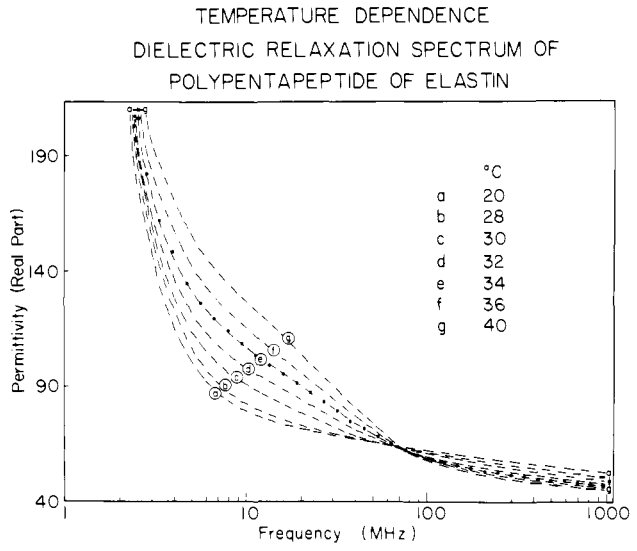

Figure 1. Real part of the dielectric permittivity, ϵ', of the coacervate of the polypentapeptide of elastin over the frequency range of 1–1000 MHz and for the temperature range of 20–40 °C. The data points are included on the 34 °C curve (curve e) to demonstrate frequencies utilized. Note the development of an intense relaxation near 10 MHz and the associated decrease in permittivity at 1000 MHz.

wherein type II Pro[2]-Gly[3] β-turns, involving a Val[4]-N-H···O-C-Val[1] hydrogen bond, function as spacers between turns of the helix and wherein a Val[4]-Gly[5]-Val[1] suspended chain segment can exhibit large amplitude, relatively low-frequency rocking motions of the peptide moieties, particularly when utilizing the inherent flexibility of the β-turn. This rocking motion has been termed peptide libration,[12,13] which derives from the root word libra, meaning scales or balance, and which is analogous to the slow rocking or oscillatory motion of a balance near equilibrium. Such a description suggests dielectric relaxation studies to determine if such motion can occur with a characteristic frequency in a sequential polypeptide as might be expected for the polypentapeptide in a β-spiral conformation. In the present report dielectric permittivity studies have been carried out in the 1 MHz–1 GHz frequency range and over the 20–40 °C temperature range. On raising the temperature, an intense simple Debye relaxation develops near 25 MHz. As the system being characterized contains only polypentapeptide and water with there being less than 70% water by volume, the relaxation is rather simply assignable to a peptide librational mode.

Dielectric measurements of the complex relative permittivity, ϵ, i.e.,

$$\epsilon(f) = \epsilon'(f) - i\epsilon''(f)$$

were carried out as a function of frequency, f, by means of a coaxial line cell/vector analyzer method. The dielectric cell and the methods are described elsewhere.[14,15] The experimental error is less than 1% in ϵ' and less than 2% in ϵ'' for frequencies greater than about 3 MHz. The cell was filled with the sample solution at 20 °C and the permittivity was measured at this temperature. Measurements were repeated at 40 °C. The sample was retained in the cell for a 24-h period at 40 °C and the measurements were repeated covering the temperature range from 40 to 20 °C. The sample was allowed to equilibrate at least 30 min after each temperature had been reached. The accuracy of the temperature was 0.1 °C.

At 20 °C the frequency spectrum of the real part of the permittivity, ϵ', shows two significant features (see Figure 1). At low frequency (f = 1–10 MHz), ϵ' steeply decreases with increasing frequency. At higher frequency (f = 10–1000 MHz), ϵ' continues to decrease but the slope is distinctly less. The low-frequency dispersion at 20 °C may be due partly to electrode polarization. After the initial heating, the 20 and 40 °C data were very reproducible. With increasing temperature an additional dispersion emerges in the range of 2–100 MHz, the increment of which increases with temperature. When the permittivity at T = 20 °C is subtracted from each curve, the resulting difference curves, i.e., $\epsilon'(f,T) - \epsilon'(f, 20\ °C)$, can be fitted by a single Cole–Cole relaxation function.[16] The values of the fitted relaxation time distribution parameter, α, are very small. This means that the relaxation is essentially of the Debye type[17] being characterized by single relaxation time.

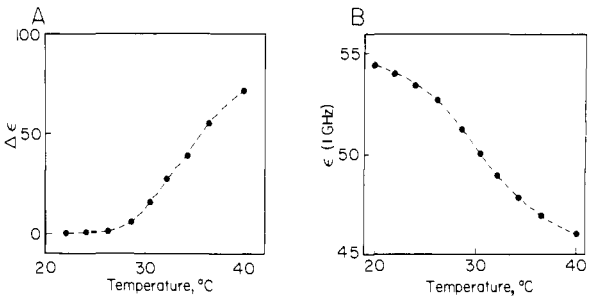

Figure 2. (A) Dielectric increment, $\Delta\epsilon$, for the relaxation having a correlation time of about 6 ns given as a function of temperature. Note the very large magnitude of $\Delta\epsilon$ at 40 °C, about 70. The values were obtained from fitting by a Cole–Cole expression in which the distribution parameter α was essentially zero. (B) Plot of the real part of the dielectric permittivity at 1000 MHz (1 GHz) as a function of temperature.

The fitted dielectric increment, $\Delta\epsilon$, increases with increasing temperature (see Figure 2A). The relaxation time is about 6 ns ($f \simeq 25$ MHz) and does not change significantly with temperature. The high-frequency permittivity, ϵ'_∞, which is nearly the value of ϵ' at 1000 MHz, decreases with increasing temperature (see Figure 2B). The ϵ_∞ reflects mainly the permittivity contribution of the water solvent. The decrease with increasing temperature, however, is greater than the temperature dependence of the water permittivity alone, as though there is a loss of higher frequency peptide or bound water contributions.

Having observed an intense simple Debye relaxation at 25 MHz, the issue of assignment is now considered. In this two-component system of polypentapeptide and water, there are no discrete charges, the polymer is neutral, the side chains of the polymer are aliphatic, and the only dipolar elements in the system are the water molecules and the peptide moieties. Of course the absence of the relaxation at 20 °C with a slightly lower water content at 40 °C where the relaxation is intense makes waters of hydration questionable as the source. The relaxation frequencies for waters of hydration are generally reported in the 1–10-GHz range,[18-22] orders of magnitude higher than the frequency of interest here. Nuclear magnetic resonance studies place the correlation time for water most tightly bound to protein to be shorter than nanoseconds and reasonably shorter than 0.1 ns.[23] Accordingly, the water component of this system is reasonably eliminated as a direct source for the observed relaxation. Whole molecule reorientation frequencies for even compact globular molecules of lower molecular weight, e.g., 65 000-dalton hemoglobins in rela-

(12) Urry, D. W.; Venkatachalam, C. M.; Long, M. M.; Prasad, K. U. *Conform. Biol.* **1983**, 11–27.
(13) Urry, D. W.; Trapane, T. L.; Long, M. M.; Prasad, K. U. *J. Chem. Soc. Faraday Trans 1* **1983**, *79*, 853–868.
(14) Henze, R.; Schreiber, U. *Ber. Bunsenges. Phys. Chem.*, in press.
(15) Göttmann, O.; Dittrich, A. *J. Phys. E.* **1984**, *17*, 772.
(16) Cole, K. S.; Cole, R. H. *J. Chem. Phys.* **1941**, *9*, 341–351.
(17) Frohlich, H. "Theory of Dielectrics", 2nd ed.; Clarendon Press: Oxford, 1958.
(18) Takashima, S.; Gabriel, C.; Sheppard, R. J.; Grant, E. H. *Biophys. J.* **1984**, *46*, 29–34.
(19) Kaatze, U.; Göttman, O.; Padbielski, R.; Pottel, R.; Terveer, U. *J. Phys. Chem.* **1978**, *82*, 112–120.
(20) Essex, C. G.; Grant, E. H.; Sheppard, R. J.; South, G. P.; Symonds, M. S.; Mills, G. L.; Slack, J. *Ann. N. Y. Acad. Sci.* **1977**, *303*, 142–155.
(21) Epstein, B. R.; Foster, K. R.; Mackay, R. A. *J. Colloid Interface Sci.* **1983**, *95*, 218–227.
(22) Foster, K. R.; Epstein, B. R.; Jenin, P. C.; Mackay, R. A. *J. Colloid Interface Sci.* **1982**, *88* 233–246.
(23) Bryant, R. G.; Shirley, W. M. *Biophys. J.* **1980**, *32*, (1), 3–16.

tively dilute solution, have characteristic frequencies of the order of 0.2 kHz.[24] Accordingly, whole molecule reorientation can be eliminated as a source for this greater than 100 000-dalton polymer at its very viscous coacervate concentration where there is less than 70% water. This leaves the rocking motion of the peptide moieties as the source of the 25-MHz relaxation. Direct nuclear magnetic relaxation studies on peptide carbonyls of elastic fiber[25] containing the polypentapeptide and of the polypentapeptide coacervate itself[26,27] provide estimates in the 10-ns range for the carbonyl carbon correlation time. The relaxation, therefore, is readily assigned to a peptide libration mode in the polypentapeptide of elastin.

The peptide librational mode builds in intensity (see Figure 2A) over the same temperature range that has been identified as the interval wherein an inverse temperature transition occurs which leads to an increase in order of the polypentapeptide with increases in temperatre.[28] This, of course, is confirmed by the present data. Rather than a broad distribution of correlation times characteristic of a distribution of conformations, a single correlation time is found with the relaxation being well fit by the Debye equation or by a single Cole–Cole term in which $\alpha = 0$. In fact the drop in the background curve with increasing temperature, followed as ϵ(1 GHz) vs. temperature (see Figure 2B), taken together with the simultaneous building of intensity of the relaxation near 25 MHz, becomes a new demonstration of an inverse temperature transition. Below 20 °C, the perspective is one of pentamers that exhibit rocking motions with a range of different correlation times derived from a dispersity of conformational states. Above 30 °C, the pentamers become arranged in a regular manner to give a single characteristic correlation time. The single correlation time represents a common dynamic conformational state for the pentamers. Therefore the polypentapeptide is describable as a regular, nonrandom array of pentamers at 40 °C.

To our knowledge this is the first demonstration of a peptide librational mode and its relevance to the entropic component of the elastomeric force of cross-linked polypentapeptide will treated in a future report.

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(24) Delalic, Z. Takashima, S.; Adachi, K.; Asakura, T. *J. Mol. Biol.* **1983**, *168*, 659–671.
(25) Lyerla, J. R., Jr.; Torchia, D. A. *Biochemistry* **1975**, *14*, 5175–5183.
(26) Urry, D. W.; Long, M. M. *Adv. Exp. Med. Biol.* **1977**, *79*, 685–714.
(27) Urry, D. W.; Trapane, T. L.; Iqbal, M.; Venkatachalam, C. M.; Prasad, K. U. *Biochemistry*, in press.
(28) Urry, D. W. *Methods Enzymol.* **1982**, *82*, 673–716.

Stereochemical Control of Yeast Reductions. 5. Characterization of the Oxidoreductases Involved in the Reduction of β-Keto Esters[1]

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Recently, we demonstrated the feasibility of altering the stereochemical course of reduction of 4-chloroacetoacetic esters by bakers' yeast via modification of the size of the ester substituent.[2] In this paper, we examine the underlying factors governing this striking stereochemical observation. We now report the isolation of three dominant competing β-keto oxidoreductases and show a distinct correlation of the ester substituent with the specificity constant,[3,4] k_{cat}/K. Moreover, ethyl (*R*)-4-chloro-3-hydroxybutanoate (**5**) of high optical purity (ee = 0.90) may now be prepared by using a mutant of *S. cerevisiae* lacking one of the competing enzymes of opposite stereochemical preference.

Table I. Kinetic Constants of β-Keto Reductases of Bakers' Yeast

$ClCH_2C(=O)$-$CH_2C(=O)OR$	fatty acid synthetase		D enzyme		L enzyme	
	K, mM	k_{cat}, s^{-1}	*K*, mM	k_{cat}, s^{-1}	*K*, mM	k_{cat}, s^{-1}
R = C_2H_5	1.82	303	1.00	0.21	1.00	6.60
R = C_4H_9	1.33	202	0.10	0.11	0.094	7.13
R = C_6H_{13}	1.82	252	0.20	0.23	0.028	6.87
R = C_8H_{17}	1.60	69	0.29	0.47	0.01	6.12

We have purified three enzymes to homogeneity from the cytosolic fraction of bakers' yeast (Red Star) capable of actively reducing 4-chloroacetoacetic esters to yield corresponding carbinolic products of high optical purity[5] (>0.97 ee). All of them utilize NADPH preferentially as the coenzyme. One of these enzymes (MW 2 400 000) possesses physical and chemical properties reminiscent of fatty acid synthetase[6] and reduces β-keto esters to yield carbinols of D configuration.[7] Although the natural substrates for the other two enzymes have not yet been defined they may be readily resolved on a hydroxyapatite column. The faster moving protein (D enzyme) has an MW of 38 000 and reduces β-keto esters to yield D carbinolic products, whereas L enzyme (MW 74 000) affords carbinols of L configuration. Because mammalian L-3-(hydroxyacyl)-CoA dehydrogenase[8] (EC 1:1:35) in the presence of NADH reduced 4-chloroacetoacetic esters to (*R*)-4-chloro-3-hydroxybutanoates of high enantiomeric excess (>0.97), we carefully searched for this reductase activity in bakers' yeast. While mitochondrial fractions of bakers' yeast actively reduced acetoacetyl-CoA, only a trace of reductase activity was detectable using either **1** or **3** as substrates. This observation indicates that L-3-(hydroxyacyl)-CoA dehydrogenases of different species have marked differences in substrate specificities.[9]

To gain an insight into the influence of the ester substituent on the enantioselective reduction of γ-chloro-β-keto esters by intact bakers' yeast, the k_{cat} (turnover number) and *K* (Michaelis constant) for the three enzymes on various 4-chloroacetoacetic esters were measured (Table I).

A moderate decrease in k_{cat}/K[4] is noted for fatty acid synthetase as the ester grouping is enlarged (Figure 1). The more pronounced drop in k_{cat}/K for the octyl ester is attributable to a decrease in the value of k_{cat} (Table I). More dramatic is the increase in the value of k_{cat}/K as the ester grouping is changed from ethyl to octyl for the L enzyme (Figure 1). Interestingly, this increase is due to a decrease in *K* (Table I). These complementary relationships between ester substituents and specificity constants for the two competing enzymes are compatible with the observed stereochemical results of resting bakers' yeasts. That is, **1** is reduced predominantly to **2** whereas **3** is reduced to **4**.

(1) For part 4 of this series, see: Sih, C, J.; Zhou, B. N.; Gopalan, A. S.; Shieh, W. R.; VanMiddlesworth, F. *Sel., Goal. Synth. Effic., Proc. Workshop Conf. Hoechst. 14th*, **1983**, *184*, 251.

(2) Zhou, B. N.; Gopalan, A. S.; VanMiddlesworth, F.; Shieh, W. R.; Sih, C. J. *J. Am. Chem. Soc.* **1983**, *105*, 5925.
(3) Sih, C. J.; Chen, C. S. *Angew. Chem., Int. Ed. Engl.* **1984**, *23*, 570.
(4) For a lucid presentation of the relationship between substrate structure and k_{cat}/K, see: Bender, M. L.; Kézdy, F. J. *Annu. Rev. Biochem.* **1965**, *34*, 49.
(5) The optically active 4-chloro-3-hydroxybutanoates were reacted with (+)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride in pyridine and then analyzed by HPLC. See ref 1.
(6) Lynen, F. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **1961**, *20*, 941.
(7) Because all three enzymes reduce β-keto esters and 4-chloro-β-keto esters, the L and D designation is preferred.
(8) Sigma H4626, type III from porcine heart.
(9) Yeast alcohol dehydrogenase in the presence of NADH is unable to reduce ethyl 4-chloroacetoacetate, whereas horse liver alcohol dehydrogenase readily reduced this substrate.

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