the high density lipoproteins (density 1.063 to 1.21). Preliminary studies in this Laboratory with this lipoprotein fraction,[30] indicate that the effect of salt upon the distribution of fatty acids between high density lipoprotein and serum albumin is similar to the effects described here for low density lipoproteins and that, in fact, only a small fraction of the plasma unesterified fatty acid is bound to high density lipoprotein. The previous observations are probably largely artifacts resulting from the very high salt concentrations used in separating the high density lipoproteins.

The data presented in Figs. 1 through 4 demonstrate that as $\bar{\nu}$ to albumin increases relatively more and more of the fatty acid becomes bound to lipoprotein. At high values of $\bar{\nu}$ to albumin a much larger percentage of the total fatty acid is therefore bound to lipoprotein. This situation exists in pathological clinical conditions, such as the nephrotic syndrome, where the serum albumin level is abnormally low, the lipoprotein concentration elevated and the unesterified fatty acid concentration normal. Under such conditions a much larger percentage of the plasma unesterified fatty acid will exist bound to lipoprotein. It is conceivable that the abnormal distribution of fatty acids might affect the metabolism of lipoproteins and/or unesterified fatty acids and hence might be involved in the metabolic abnormalities of these conditions.

(30) D. S. Goodman and E. Shafrir, unpublished observations.

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[CONTRIBUTION FROM THE LABORATORY OF THE CHILDREN'S CANCER RESEARCH FOUNDATION]

The Preparation of High Molecular Weight Polypeptides[1]

BY E. R. BLOUT[2] AND M. E. DESROCHES

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A method is described for the preparation of polypeptides of very high molecular weight, *i.e.*, with weight average molecular weights as high as 1,000,000. The method involves intermolecular condensation of lower molecular weight polypeptides using carbodiimides as the condensing agents. The method has been used with both organo-soluble polypeptides and water-soluble polypeptides and to prepare block copolymers. Examples are given of the preparation of high molecular weight poly-γ-benzyl-L-glutamate, poly-ε-carbobenzyloxy-L-lysine and poly-L-proline.

Polypeptides, or poly-α-amino acids, are useful models for studies of the chemical and biological properties of proteins. For these purposes it is necessary that the polypeptides have molecular weights comparable to those of proteins, *i.e.*, 50,000 to 1,000,000. Using the strong base initiated polymerization of α-amino acid-N-carboxyanhydrides (NCAs), it has been possible to prepare polypeptides in this molecular weight range from glutamic esters,[3] lysine,[4] proline[5] and and other amino acids.[6] However, with certain amino acids only lower molecular weight polypeptides (molecular weights of 10,000 to 35,000) have been synthesized. In this paper we describe a method for obtaining high molecular weight polypeptides by joining together lower molecular weight fragments.

The method makes use of the carbodiimide reagent introduced by Sheehan and Hess for the formation of peptide bonds.[7] If a polypeptide with free terminal amino and carboxyl groups is treated with a carbodiimide, then condensation may occur with formation of additional peptide bonds between polymers to yield products of increased molecular weight and degree of polymerization (DP) in the manner indicated below

$$\begin{aligned}&\mathrm{H_2N\text{-}Polypeptide\text{-}COOH} + \underset{(DP_x)}{\quad}\\ &\qquad \mathrm{H_2N\text{-}Polypeptide\text{-}COOH} + \mathrm{R{-}N{=}C{=}N{-}R'} \quad (DP_y)\\ &\longrightarrow \mathrm{R{-}N{-}\overset{\overset{O}{\|}}{C}{-}N{-}R'} + \mathrm{H_2N\text{-}Polypeptide\text{-}COOH} \longrightarrow \underset{(DP_{2x}) \text{ and } (DP_{2y})}{(DP_{x+y}),}\\ &\qquad\qquad \mathrm{H_2N\text{-}Polypeptide\text{-}COOH}\\ &\qquad\qquad (DP_{2(x+y)}),\ (DP_{2x+y}),\ (DP_{x+2y}),\ (DP_{3x}) \text{ and } (DP_{3y}) \longrightarrow \text{etc.}\end{aligned}$$

It is apparent that the polypeptides indicated above may be derived from the same amino acid or different amino acids. If the latter is the case then this procedure will produce "block" polypeptides. The results obtained with polypeptides derived from four different amino acids are shown in the Table I.

It is clear from the data in the table that through the use of carbodiimides as condensing agents the molecular weights of polypeptides can be increased significantly. In several instances fourfold or greater increases have been observed.

It is assumed that this increase in molecular weight occurs because of terminal inter-molecular peptide bond formation, but definitive proof is difficult to obtain. However, indirect evidence that linear peptide bond formation is involved can be deduced from the fact that we have observed that polypeptides with one terminal amide group and one terminal amine group do not undergo molecular weight increases when treated with car-

(1) This paper is Polypeptides. XXIV. For the previous paper in this series see H. Lenormant, M. Baudras and E. R. Blout, THIS JOURNAL, **80**, 6191 (1958).

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(3) (a) E. R. Blout, R. H. Karlson, P. Doty and B. Hargitay, THIS JOURNAL, **76**, 4492 (1954); (b) E. R. Blout and R. H. Karlson, *ibid.*, **78**, 941 (1956); (c) M. Idelson and E. R. Blout, *ibid.*, **80**, 2387 (1958).

(4) (a) E. R. Blout and H. Lenormant, *Nature*, **179**, 960 (1957); (b) E. R. Blout and M. Idelson, unpublished results.

(5) E. R. Blout and G. D. Fasman, "Recent Advances in Gelatin and Glue Research," Pergamon Press, London, 1957, p. 122.

(6) E. R. Blout and co-workers, unpublished results.

(7) J. C. Sheehan and G. D. Hess, THIS JOURNAL, **77**, 1067 (1955).

TABLE I

Starting polypeptide[a]	$(\eta_{sp/c})^b$	MW_w[c]	Solvent[d]	Final polypeptide $(\eta_{sp/c})^c$	Final polypeptide MW_w[c]	Preparation no.
Poly-γ-benzyl-L-glutamate	0.96	155,000	Dioxane	3.10	580,000	427
Poly-γ-benzyl-L-glutamate	0.96	155,000	Dioxane	3.35	630,000	440
Poly-γ-benzyl-L-glutamate	1.22	200,000	Dioxane	4.82	950,000	474
*Poly-γ-benzyl-L-glutamate	1.33	225,000	Dioxane	4.21	820,000	480
*Poly-γ-benzyl-L-glutamate	1.33	225,000	Chloroform	4.96	1,000,000	483
Poly-ε-carbobenzyloxy-L-lysine	1.71	350,000	Chloroform	3.70	840,000	482
+Poly-ε-carbobenzyloxy-L-lysine	0.17	17,000	Chloroform	0.19	20,000	F88-2
+Poly-ε-carbobenzyloxy L-lysine	0.43	60,000	Chloroform	0.45	63,000	F88-1
*Poly-L-proline	0.18	10,000	Water	0.32 (c = 0.14%)	32,000	527
*Poly-L-proline	1.63	122,000	Water	1.89	146,000	507
Poly-γ-benzyl-L-glutamate and Poly-ε-carbobenzyloxy-L-lysine in 1:1 ratio	1.22 1.50	200,000 300,000	Chloroform	2.99	600,000	487
Poly-γ-benzyl-L-glutamate and Poly-ε-carbobenzyloxy-L-lysine in 1:1 ratio	1.23 0.72	200,000 132,000	Chloroform	1.49	280,000	537

[a] In all cases the starting polypeptides were homopolymers, *i.e.*, composed of only one amino acid. Where indicated mixtures of two homopolymers reacted. The starting polypeptides were all initiated with NaOH in water except where indicated by * or +. *, starting polypeptides initiated with $NaOCH_3$ in methanol:benzene; +, starting polypeptide initiated with *n*-hexylamine. [b] All viscosities were measured in dichloroacetic acid. Except where indicated, c = 0.2%. [c] The weight average molecular weights (MW_w) were estimated from the viscosity data[3b] using the calibration with light scattering measurements for polybenzyl-L-glutamate provided by P. Doty, J. H. Bradbury and A. M. Holtzer, THIS JOURNAL, **78**, 947 (1956), and J. C. Mitchell, A. E. Woodward and P. Doty, *ibid.*, **79**, 3955 (1957). [d] Dicyclohexylcarbodiimide was used as the condensing agent in all the organic solvents. 1-Ethyl-3-[2-morpholinyl-(4)-ethyl]-carbodiimide methotoluenesulfonate (J. C. Sheehan and J. J. Hlavka, *J. Org. Chem.*, **21**, 439 (1956)) was used with the poly-L-proline in aqueous solution. We are much indebted to Dr. John Sheehan for supplying us with a sample of this compound.

bodiimides. For example when poly-ε-carbobenzyloxylysine, prepared by amine initiation and thus containing a terminal amide group[8] per molecule, was treated in the manner described in the experimental section IIA, the observed change in molecular weight was within the limits of error in the measurements (see preparations #F88–2 and F88–1 in Table). Thus it may be concluded that the carbodiimide reaction with polypeptides will not proceed when the carboxyl end of the chain is blocked by amide formation. These experiments provide indirect evidence that the molecular weight increases observed with other polypeptide preparations is due to linear peptide bond formation.

When this work was started, it was thought that for terminal intermolecular condensations it would be necessary for the starting polypeptides to contain both free terminal carboxyl and amino groups. Therefore the NCAs were initiated with aqueous sodium hydroxide solution which should lead to polypeptides containing terminal carboxyl and amino groups. During the course of the work, however, it was found that initiation with sodium methoxide[3b] also yielded polypeptides which underwent interchain condensation in the presence of carbodiimides. In both methoxide and hydroxide initiation it is assumed that free terminal amino groups are present. Three possible explanations have been considered to explain the methoxide initiated polymer results. First, that both terminal carboxyl groups and terminal–$COOCH_3$ groups are present in methoxide initiated polymers, and only the carboxyl terminal polymers participate in the carbodiimide reaction. Because of the high molecular weight of the polypeptides, titration for free carboxyl groups in these starting polypeptides (designated by an asterisk in the table) has not allowed the unequivocal determination of their carboxyl content. Secondly, it is possible that methanol rather than water is removed by the carbodiimide. Thirdly, it is possible that intermolecular condensations other than terminal ones have occurred. We consider this last suggestion unlikely both because of the experiments cited above in which no molecular weight increase was observed when a terminal group of the polypeptide was amide and also because of the poly-L-proline results. With this polypeptide no intermolecular condensations other than terminal may occur and poly-L-proline does show a definite molecular weight increase when treated with carbodiimides.

From the experiments we have performed it is evident that this method can be applied to prepare polypeptide polymers not easily made otherwise. For example, the carbodiimide method should make possible the preparation of high molecular weight D,L-polypeptides and high molecular weight water soluble polypeptides which have not been prepared directly from water soluble NCAs.[9,10] In addition, using the carbodiimide method it is possible to prepare "block" copolymers from two or more lower molecular weight polypeptides (*cf.* the table). It is recognized that such block polypeptides will have a random arrangement of "blocks." If the number of blocks joined together is large enough, only heteropolymers should result. Finally mention may be made that this method lends itself to the preparation of branched or multichain polypeptides[11] of

(8) See for example M. Idelson and E. R. Blout, THIS JOURNAL, **79**, 3948 (1957), and previous references cited therein.

(9) R. R. Becker and M. A. Stahmann, *ibid.*, **76**, 3707 (1954).

(10) (a) P. D. Bartlett and R. H. Jones, *ibid.*, **79**, 2153 (1957); (b) P. D. Bartlett and D. C. Dittmer, *ibid.*, **79**, 2159 (1957).

(11) M. Sela and E. Katchalski, *Experientia*, XI, 62 (1955); M. Sela, E. Katchalski and M. Gehatia, THIS JOURNAL, **78**, 746 (1956).

high molecular weight and also to increasing the molecular weight of many types of polymers having free amino and carboxyl groups.[12]

Experimental

I. Preparation of Starting Polypeptides.—**A. Poly-γ-benzyl-L-glutamate (sample 474).**—2.0 g. of γ-benzyl-L-glutamate-N-carboxy anhydride[3b] was added to 50 cc. of freshly distilled dioxane. To this solution was added 183 cc. of 0.416 *N* NaOH at −30°. This corresponds to an anhydride:initiator ratio (A/I) of 100. The stoppered flask was allowed to stand at room temperature for 2 hr. during which time the solution became viscous.[13] The polymer was isolated by pouring the above solution into approximately seven times its volume of 95% ethanol which contained sufficient HCl to neutralize the initiator. It was then filtered, washed with anhydrous ether, dried *in vacuo* and stored at 2°. The yield was quantitative; $\eta_{sp/c}$ (*c* 0.2%, in DCA) = 1.22.

Samples number 480 and 483 were prepared using $NaOCH_3$ in benzene–methanol (3:1) as initiator for the polymerization.[3b]

B. Poly-ε-carbobenzyloxy-L-lysine was prepared from the corresponding NCA[14] in the manner described above.

C. Poly-L-proline was prepared from L-proline NCA[15] using $NaOCH_3$ initiation.

II. Preparation of High Molecular Weight Polypeptides.—The following are typical procedures.

A. In Organic Solvents.—0.2 g. of poly-γ-benzyl-L-glutamate ($\eta_{sp/c}$ = 1.22) was dissolved in 20 cc. of dioxane and 0.1 g. of dicyclohexylcarbodiimide was added which dissolved immediately. The reaction mixture was allowed to stand at room temperature for 24 hr. during which time the solution became more viscous. The polymer then was precipitated by pouring the reaction mixture into 100 cc. of 95% ethanol; the dicyclohexylurea and dicyclohexylcarbodiimide is soluble in the ethanol whereas the polymer is insoluble. The polymer then was dried at 100° *in vacuo* for 12 hr. The yield was quantitative: $\eta_{sp/c}$ = 4.82 (*c* 0.2%, in DCA).

B. Aqueous Solutions.—0.028 g. of the starting poly-L-proline ($\eta_{sp/c}$ (*c* 0.2%, in DCA) = 0.18) was dissolved in 5 cc. of distilled water, and 0.020 g. of 1-ethyl-3-[2-morpholinyl-(4)-ethyl]-carbodiimide metho-*p*-toluenesulfonate was added. The reaction mixture was allowed to stand at room temperature for 24 hr. The solvent was removed by lyophilization and the polymer was extracted with absolute ethanol for 2 hr. to remove the substituted urea and the unreacted carbodiimide. After drying at 100° *in vacuo* $\eta_{sp/c}$ (*c* 0.14%, in DCA) = 0.32.

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(12) *Cf.* J. C. Sheehan and J. J. Hlavka, THIS JOURNAL, **79**, 4258 (1957).

(13) In the poly-γ-benzyl-L-glutamate preparations the polymerization was stopped soon after the consumption of the NCA and the polymers were stored at 2° in order to minimize the formation of pyrrolidones at the terminal amino acid residues (W. E. Hanby, S. G. Waley and J. Watson, *J. Chem. Soc.*, 3239 (1950)) which would interfere with the subsequent carbodiimide condensations.

(14) M. Bergmann, L. Zervas and W. F. Ross, *J. Biol. Chem.*, **111**, 245 (1935).

(15) A. Berger, J. Kurtz and E. Katchalski, THIS JOURNAL, **76**, 5552 (1954).

BOSTON 15, MASS.

[CONTRIBUTION FROM THE DEPARTMENT OF ENTOMOLOGY OF THE UNIVERSITY OF CALIFORNIA CITRUS EXPERIMENT STATION]

The Effect of Structure on the Reactivity of Alkylphosphonate Esters[1,2]

BY T. R. FUKUTO AND R. L. METCALF

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A number of new ethyl *p*-nitrophenyl alkylphosphonates were prepared and the effect of the alkyl moiety on the lability of the phosphorus–O–*p*-nitrophenyl bond as measured by alkaline hydrolysis was examined. It was found that the rate of hydrolysis of these alkylphosphonates to *p*-nitrophenol and ethyl alkylphosphonic acid, in general, decreased with increase in alkyl chain length. Compared to the straight chain compounds, branching in the 1- and 2-positions greatly decreased the hydrolysis rate. The rate of reaction of these compounds with insect cholinesterase also was measured and compared with the hydrolysis rate. Many of these compounds showed high toxicity to the common house fly, *Musca domestica* L., and degree of toxicity was parallel to cholinesterase inhibition.

The toxicity of certain organophosphorus compounds to mammals and insects has generally been associated with the inhibition of the cholinesterase (ChE) enzymes. The inhibition of these esterases by organophosphorus compounds has clearly been demonstrated to be the result of an actual chemical reaction between the enzyme and the phosphorus compound.[3] The phosphorylated enzyme thus produced is no longer able to catalyze the hydrolysis of certain esters.

The inhibition of erythrocyte cholinesterase by diethyl *p*-nitrophenyl phosphate (para-oxon) and some of its analogs has been shown to proceed with pseudo first-order kinetics.[4] The bimolecular rate constants for this inhibition were determined for the various para-oxon analogs, and these values paralleled the rates of hydrolysis of these phosphates in water. More recently[5] it was demonstrated from the study of a large series of diethyl-substituted phenyl phosphates that the inhibition of fly-head cholinesterase by these compounds was related to the effect of the substituent on the lability of the P–O–phenyl bond as measured by Hammett's σ constants, shifts in P–O–phenyl stretching frequencies, and hydrolysis rates.

Although the phosphate esters and their sulfur analogs have been widely examined as inhibitors of cholinesterase and evaluated as insecticides, relatively little has been published on the activity of phosphonate esters in spite of the fact that some of the most potent inhibitors are derivatives of phosphonate esters. 3,3-Dimethyl-2-butyl methylphos-

(1) Paper No. 1052, University of California Citrus Experiment Station, Riverside, Calif.

(2) Supported in part by a research grant from the Monsanto Chemical Company, St. Louis, Mo.

(3) T. R. Fukuto, in "Advances in Pest Control Research," Vol. I, Interscience Publishers, Inc., New York, N. Y., 1957, pp. 147–192.

(4) W. N. Aldridge and A. N. Davison, *Biochem. J.*, **51**, 62 (1952).

(5) T. R. Fukuto and R. L. Metcalf, *J. Agr. Food Chem.*, **4**, 930 (1956).