reactions,[3,4,5] and (c) show strong molecular weight and rate dependence on the optical isomer composition of the anhydride.

The reaction rates were determined at 4% anhydride concentration in dioxane solution at 31 ± 1° by measuring the disappearance of the 1860 and 1790 cm.$^{-1}$ anhydride carbonyl frequencies during the polymerization.[5] Four BLGA rate curves were obtained for anhydride:initiator (A/I) mole ratios of 40, 100, 400 and 1000. At each A/I the initial portion of the reaction is autocatalytic but, after the reaction is about one-third completed, it becomes first order in anhydride concentration. The times for 90% completion of the reactions for the above A/I's are 30, 53, 108 and 180 minutes, respectively. Similar data have been obtained with other amino acid anhydrides and other solvents. The propagation rate constants, k_p, were calculated from the first order portion of the curves using the equation

$$k_p = \frac{\ln \frac{OD_2}{OD_1} \times DP_w}{(t_2 - t_1)A_0}$$

where OD = optical density of the anhydride carbonyl band, t = time in seconds, A_0 = initial anhydride concentration, and DP_w = weight average degree of polymerization of the final polymer. It is necessary to employ the degree of polymerization since only a fraction of the anions start growing chains. This is shown by the fact that, in the range of A/I's between 50 and 1000, the DP_w is always much higher than the A/I.[2] Inasmuch as the polydispersity of PBLG prepared in this manner has been shown to be less than 2,[6] the use of A_0/DP_w is a reasonable approximation of the actual growing chain concentration. The experimental results indicate that, over a twenty-five fold range of A/I, k_p = 6.2 ± 0.6 liter/mole/sec. This rate constant is about 100 times that found for amine initiation.

Since it is known that sodium methoxide reacts rapidly and quantitatively with N-carboxyanhydrides to yield two products,[7,2] and since the strong base initiated polymerization is not first order during the early part of the reaction, sodium methoxide cannot be the true initiator. The autocatalytic nature of the kinetic curve suggests that one or both of the primary reaction products are subsequently and more slowly converted to the true initiating species. Support for this suggestion is obtained from the longer autocatalytic periods observed as the sodium methoxide concentration is decreased, *i.e.*, at higher A/I's. The effect of initiating with each of the two primary products of the reaction of sodium methoxide and anhydride is being investigated.

Polymerizations of BLGA have been performed using sodium triphenylmethyl in ether as the primary initiator. The reaction rate data were essentially the same as for sodium methoxide (in methanol) initiated reactions indicating that a labile proton on the initiator or the solvent is not required. Initiation by strong bases is evidently quite different from salt-initiated polymerizations described by Ballard and Bamford.[8] With base initiation the rate increases during the course of the reaction whereas in salt-initiated polymerizations the observed rate is slower and decreases further during the reaction.

When γ-benzyl-N-carboxy-D,L-glutamate anhydride is polymerized with $NaOCH_3$ initiation the reaction rate constant is 0.36—approximately $^1/_{20}$ that observed with both the pure D and L isomers. Reaction rate and molecular weight data for various mixtures of D and L anhydrides are shown in the table.

POLYMERIZATIONS OF γ-BENZYLGLUTAMATE-N-CARBOXYANHYDRIDES AT A/I = 100 IN DIOXANE

% D isomer	0	5	25	50	90	95	100
k_p	6.1	2.1	0.37	0.36	0.97	2.0	6.0
DP_w	900	580	275	200	515	525	800

It is apparent that the presence of the opposite optical isomer has an effect *far beyond* that which would be predicted on a simple *infinite preference* of a growing chain of one isomer for its own isomer, since this should only diminish the rate by $^1/_2$. Interesting possibilities that would explain the experimental rate and molecular weight results with mixtures of optical isomers are (a) the anhydride complexes strongly with its opposite isomer or with growing chains of the opposite configuration, and (b) the polypeptide helices which are formed have a preferred screw direction for each optical isomer and the rate of incorporation of the opposite isomer is lower due to steric interferences.[9]

(3) D. G. H. Ballard and C. H. Bamford, *Proc. Roy. Soc.* (*London*) **A233**, 495 (1954).
(4) P. M. Doty and R. L. Lundberg, to be published.
(5) M. Idelson and E. R. Blout, to be published.
(6) J. T. Yang and P. Doty, personal communication.
(7) A. Berger, M. Sela, and E. Katchalski, *Anal. Chem.*, **25**, 1554 (1953).
(8) D. G. H. Ballard and C. H. Bamford, Symposium of Peptide Chemistry Special Publication No. 2, The Chemical Society, London, p. 25 (1955).
(9) This work was supported by the Office of the Surgeon General, Department of the Army.
(10) Chemical Research Laboratory, Polaroid Corporation, Cambridge 39, Massachusetts.

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RECEIVED JUNE 14, 1956

THE AMINO ACID SEQUENCE OF GLUCAGON

Sir:

A previous report[1] from this laboratory[2] described the isolation in crystalline form and the preliminary structure study of glucagon, the hyperglycemic-glycogenolytic hormone of the pancreas. We now wish to report the complete amino acid sequence of glucagon.[3]

Quantitative amino acid chromatography[4] and chemical analyses of tryptophan[5] and amide am-

(1) A. Staub, L. Sinn and O. K. Behrens, *J. Biol. Chem.*, **214**, 619 (1955).
(2) Biochemical Research Department, Lilly Research Laboratories, Indianapolis.
(3) We gratefully acknowledge the assistance of our associates, C. W. Pettinga, H. L. Bird, E. R. Diller, W. A. Tandy and R. Scheib.
(4) S. Moore and W. H. Stein, *J. Biol. Chem.*, **192**, 663 (1951).
(5) J. R. Spies and D. C. Chambers, *Anal. Chem.*, **20**, 30 (1948).

TABLE I

STRUCTURE OF GLUCAGON

Peptide number	Hydrolytic agent	Peptide
ST-3	Trypsin (2.25 hr.)	His(ser,glu,gly,thr,phe,thr,ser,asp,tyr,ser,lys)
S-8A	Subtilisin	His(ser,glu)
C-4	Chymotrypsin and	
LT-5A	Trypsin (50 hr.)	His.ser.glu.gly.thr.phe (NH_2 on glu)
S-6	Subtilisin	gly(thr,phe)
LT-3	Trypsin (50 hr.)	thr.ser.asp.tyr.ser.lys
S-1	Subtilisin	thr.ser
C-2	Chymotrypsin	thr(ser,asp,tyr)
S-3	Subtilisin	asp(tyr,ser)
C-6	Chymotrypsin	ser(lys(tyr)
S-10	Subtilisin	lys.tyr
ST-1	Trypsin (2.25 hr. and 50 hr.)	
LT-4		tyr.leu.asp.ser.arg
S-7	Subtilisin	leu(asp,ser,arg)
C-5	Chymotrypsin	leu(asp,ser,arg,arg,ala,glu,asp,phe)
ST-2	Trypsin (2.25 hr.	
LT-5B	and 50 hr.)	arg
S-9	Subtilisin	arg(ala,glu) (NH_2 on glu)
ST-P	Trypsin	ala(glu,asp,phe,val,glu,try,leu,met,asp,thr)
LT-2	Trypsin (50 hr.)	ala(glu,asp,phe,val,glu,try)
S-4	Subtilisin	asp.phe
C-3	Chymotrypsin	
S-8B	Subtilisin	val.glu.try (NH_2 on glu)
S-5	Subtilisin	leu.met
C-1	Chymotrypsin and	
LT-1	Trypsin (50 hr.)	leu(met,asp,thr)
S-2	Subtilisin	asp.thr (NH_2 on asp)
Amino acids from carboxypeptidase		ala(glu,asp,phe,val,glu,try,leu,met,asp)thr (NH_2 on first glu, second glu and asp)
Summary		His.ser.glu.gly.thr.phe.thr.ser.asp.tyr.ser.lys.tyr.leu.asp.ser.arg.arg.ala.glu.asp.phe.val.glu.try.leu.met.asp.thr (NH_2 on glu at position 3, glu at position 20, glu at position 24 and asp at position 28)

monia[6] provided evidence for the following empirical formula with 29 amino acid residues, and with a minimum molecular weight of 3482: $His_1Ser_4Glu_3Gly_1Thr_3Phe_2Asp_4Tyr_2Lys_1Leu_2Arg_2Ala_1Val_1Try_1Met_1(-CONH_2)_4$.[7] A molecular weight of approximately 4000 was obtained by preliminary ultracentrifugal studies utilizing a solution of glucagon in 2 *M* guanidine in the artificial boundary cell.[8] Histidine was determined as the N-terminal amino acid by means of the dinitrophenylation method; the C-terminal residue is threonine on the basis of data obtained from hydrazinolysis[9] and carboxypeptidase[10] treatment. Carboxypeptidase quantitatively liberated the following amino acids from the C-terminus of glucagon: valine, glutamine, tryptophan, leucine, methionine, asparagine, and threonine. A small amount of alanine and somewhat greater quantities of phenylalanine, aspartic acid, and a second residue of glutamine were also released.

A single lot of carefully purified crystalline glucagon was used throughout the structure studies. The methods employed in the structure determination were as follows: (a) specific enzymatic cleavage[11] with trypsin, chymotrypsin, and subtilisin[12]; (b) resolution of the peptides from these digestions using Dowex 50 chromatography; and (c) characterization of the peptides by quantitative amino acid analysis, dinitrophenylation, and in some cases further degradation with carboxypeptidase and acid.

Dowex 50 chromatography of the chymotryptic digest resulted in the separation of six pure peptide

(6) M. W. Rees, *Biochem. J.*, **40**, 632 (1946).

(7) The abbreviations used for the amino acid residues are those proposed by E. Brand and J. T. Edsall, *Ann. Rev. Biochem.*, **16**, 224 (1947).

(8) Value determined by E. O. Davisson and H. W. Fisher, Physicochemical Research Division, Lilly Research Laboratories.

(9) S. Akabori, K. Ohno and K. Narita, *Bull. Chim. Soc. Japan*, **25**, 214 (1952).

(10) Kindly supplied by Dr. Hans Neurath, University of Washington Medical School, Seattle.

(11) The possibility of enzyme-catalyzed rearrangements has been considered. Data obtained by application of several degradative methods were all consistent and failed to provide evidence for such reactions.

(12) We gratefully acknowledge receipt of subtilisin from Dr. M. Ottesen, Carlsberg Laboratories, Copenhagen.

fragments: his(ser,glu,gly,thr,phe)[13]; thr(ser,asp, tyr); ser(lys,tyr); leu(asp_2,ser,arg_2,ala,glu,phe); val(glu,try); and leu(met,asp,thr).

Four fragments were isolated from a 2.25 hour tryptic digestion of glucagon: his(ser_3,glu,thr_2,phe, gly,asp,tyr,lys); tyr(leu,asp,ser,arg); arg; and ala-(glu_2,asp_2,phe,val,try,leu,met,thr).

Six peptides were obtained from a 50-hour tryptic digest: his(ser,glu,gly,thr,phe); thr(ser_2,asp, tyr,lys); tyr(leu,asp,ser,arg); arg; ala(glu_2,asp, phe,val,try); and leu(met,asp,thr).

Subtilisin digestion followed by chromatographic resolution gave the following eleven peptides: his(ser,glu); gly(thr,phe); thr.ser; asp(tyr,ser); lys.tyr; leu(asp,ser,arg); arg(ala,glu); asp.phe; val(glu,try); leu.met; and asp.thr.

In a great majority of cases specific enzymatic splits of greater than 80% were obtained and the resulting peptides were isolated in yields exceeding 50%. With every enzyme used, the sum of the resulting fragments was in complete agreement with the empirical formula of glucagon.

The structure of peptide LT-4 was completely elucidated by partial acid degradation. Similar acid degradation and carboxypeptidase rate studies revealed the sequence in peptide LT-3. The sequence of amino acids in peptide C-4 was resolved utilizing subtilisin data and partial acid splitting.

Peptides C-3, C-4, S-2, S-3, S-4, S-7 and S-9 were selected for study of amide linkages since each contained but one such potential group. Only four peptides, C-3, C-4, S-2 and S-9, yielded stoichiometric quantities of ammonia when incubated with concentrated acid. These data are in complete agreement with the determined number of amide groups, and with the behavior of the respective peptides on ion exchange columns.

The sequence in peptide S-8B was determined by a time study with carboxypeptidase.

Integration of these data provides a basis for the complete amino acid sequence of glucagon (*cf.* Table I). The details of this work will be reported in publications now being prepared.

(13) The arrangement of peptides is patterned after that suggested by F. Sanger, *Advances in Protein Chem.*, **7**, 1 (1952).

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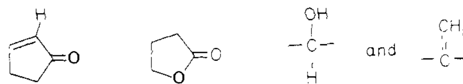
TERPENES. VI. THE STRUCTURES OF HELENALIN AND ISOHELENALIN

Sir:

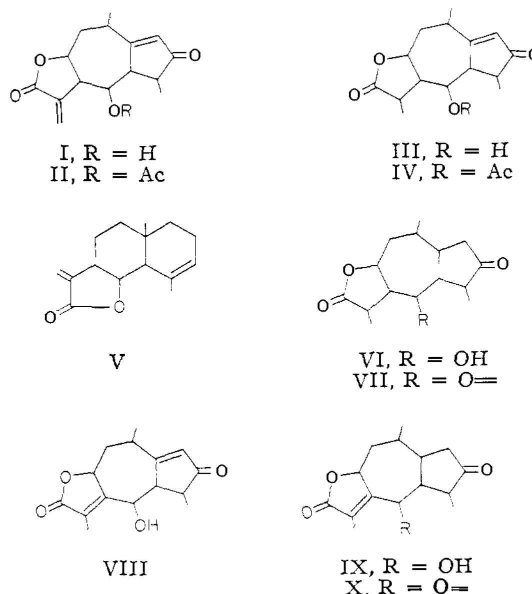
Helenalin ($C_{15}H_{18}O_4$), the active principle of the common sneezeweed, *Helenium microcephalum Linn* was first isolated by Lamson[1] from the related species, *Helenium automnale.* Structurally it did not come under close scrutiny until investigated by R. Adams and W. Herz[2] in 1949. These authors have provided conclusive evidence for the presence of the following structural units in the helenalin molecule



We now wish to discuss a few additional experiments from which we have derived expression I for this natural product. The ultraviolet spectrum of I (λ_{max} 220 mμ,ϵ 12200) is a composite of two isolated chromophores: By subtracting the curve of dihydrohelenalin (III), (λ_{max} 229 mμ,ϵ 6500) from the one of I a curve (λ 210, ϵ 10,000) resulted which is identical with the one due to the unsaturated lactone chromophore present in alantolactone (V).[3] The methylene group in I is thus conjugated with the lactone carbonyl.

It now became necessary to establish the carbon skeleton of I. Tetrahydrohelenalin (VI) was reduced with lithium aluminum hydride to a tetrol which on acetylation was converted to a tetraacetate (m.p. 86–87°, found: C, 62.78; H, 8.16). Catalytic dehydrogenation of this compound over palladium at 305° yielded guaiazulene. Helenalin (I) is therefore a sesquiterpene.

From the following interconversion it became clear that I is a γ-hydroxylactone. A second isomeric substance was present in *Helenium microcephalum* which we would like to name isohelenalin (VIII) (m.p. 260–262°, found: C, 68.69; H, 7.22; λ_{max} 219 mμ, ϵ 20,000, infrared max. in KBr 2.97; 5.76; 5.90; 6.35μ). On catalytic reduction VIII gives dihydroisohelenalin (IX) (m.p. 212°, found: C, 68.23; H, 7.60; λ_{max} 217 mμ, ϵ 14,500, infrared max. in KBr 3.02; 5.79μ) which is resistant to further hydrogenation. The corresponding diketone (X) (m.p. 150–152°, found: C, 68.78; H, 7.05, λ_{max} 234 mμ, ϵ 15,000, infrared max. 5.70; 5.74; 5.98μ) obtained by oxidation of IX with chromic acid on reduction with zinc in acetic acid yields tetrahydrohelenalone (VII).[2]



I, R = H; II, R = Ac
III, R = H; IV, R = Ac
V
VI, R = OH; VII, R = O=
VIII
IX, R = OH; X, R = O=

(1) P. D. Lamson, *J. Pharmac. Exper. Ther.*, **4**, 471 (1913).

(2) R. Adams and W. Herz, THIS JOURNAL, **71**, 2546, 2551, 2554 (1949).

(3) L. Ruzicka, P. Pieth, T. Reichstein and L. Ehmann, *Helv. Chim. Acta*, **16**, 268 (1933); W. G. Dauben and P. D. Hance, THIS JOURNAL, **75**, 3352 (1953).