reactions of *o*-phenylene carbonate with tri-*n*-butylphosphine and triethyl phosphite, the subsequent reactions of the intermediate with the nucleophiles precludes the use of this procedure for the generation of benzyne for reaction with other species.

Experimental Section

Reaction of *o*-Phenylene Carbonate (6) with Triethyl Phosphite (7).—A mixture of 6.80 g (50 mmol) of **6**, 31.5 g (250 mmol) of **7** and 0.1 g of copper dust was placed in a 100-ml Pyrex tube, which was evacuated and sealed. The reaction mixture was held at 200–205° in an oil bath for 24 hr and allowed to cool to room temperature. Glpc analysis[7] of the reaction mixture showed the presence of **7** (5%), triethyl phosphate (101%), diethyl phenylphosphonate (**8**, 22%), and triphenylene (3%) (yields are based on **6**). No unreacted **6** was detected; a large amount of diethyl ethylphosphonate, the thermal isomerization product of **7**, was present. Distillation of the reaction mixture led to the isolation of **7**, triethyl phosphate, and **8** (1.93 g, 18%), identified by comparison with an authentic sample.[7]

Registry No.—Benzyne, 462-80-6; **6**, 2171-74-6; **7**, 122-52-1.

Acknowledgment.—This study was supported in part by a grant from the Gulf Oil Foundation.

(7) For columns and conditions, see J. B. Plumb and C. E. Griffin, *J. Org. Chem.*, **28**, 2908 (1963); J. B. Plumb, R. Obrycki, and C. E. Griffin, *ibid.*, **31**, 2455 (1966).

The Synthesis of Peptides in Aqueous Medium. VI.[1] The Synthesis of an Unsymmetrical Cystine Peptide Fragment of Insulin

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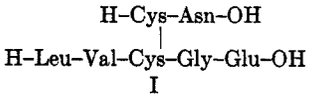
It has been reported[2] that enzymatic degradation of insulin with the enzymes of rat adipose tissue gives peptide fragments which show a high degree of insulin activity. One of the fragments reported to possess insulin activity is the sequence A chain 20–21 coupled through a disulfide bond to B chain 17–21 (I). To determine in an unequivocal manner whether this small part of the insulin molecule is indeed biologically active, we undertook the synthesis of I.



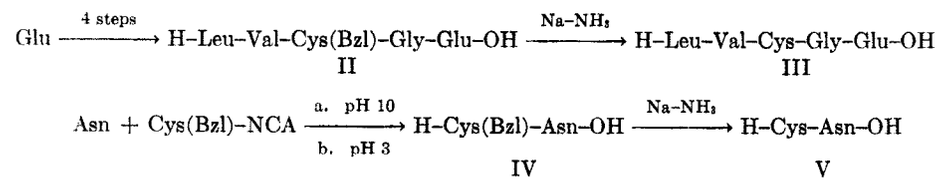
I

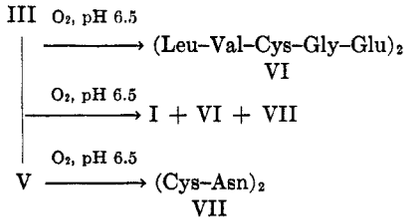
A number of methods[3] for the synthesis of unsymmetrical disulfides have been reported. The least attractive of these methods would appear to be the direct oxidation of a mixture of the two cysteine peptides, since a mixture of three peptides would be expected.[4] However, we felt that the ready availability of the dipeptide Cys–Asn would allow its use in large excess thus permitting the most effective utilization of the pentapeptide Leu–Val–Cys–Gly–Glu. We, therefore, undertook the preparation of the two necessary peptides as outlined in Scheme I.

Both peptides were prepared by the N-carboxyanhydride (NCA) procedure.[5,6] The preparation of II was carried out in four steps without isolation of intermediates, but each step was monitored by thin layer chromatography. In the preparation of Gly–Glu, we replaced the NCA of glycine by 2,5-thiazolidinedione which has been shown to afford significantly higher yields than the N-carboxyanhydride.[1] On acidification to pH 3 to dethiocarboxylate the initially formed thiocarbamoyl dipeptide, the reaction mixture was shown to contain no detectable glutamic acid and only a trace of glycine by tlc. Subsequent addition of the NCA's of S-benzylcysteine, valine, and leucine, respectively, gave II. On the final acidification (after the addition of the NCA of leucine) the crystalline product separated from the reaction medium. This material was not completely pure (minor peptide impurities were observed on tlc). The amino acid analysis was consistent with II and the material in this form was satisfactory for the subsequent preparation of pure I and VI.

The dipeptide IV was prepared by the action of S-benzylcysteine N-carboxyanhydride on asparagine. The crystalline product was identified by elemental analysis and base titration. The S-benzyl blocking groups of II and IV were removed by the action of sodium in liquid ammonia to give III[7] and V, respectively. An aliquot of each of these in aqueous solution was air oxidized at pH 6.5 to give the expected cystine peptides VI and VII, respectively. When a mixture of

SCHEME I





(1) Part V: R. S. Dewey, E. F. Schoenewaldt, H. Joshua, W. J. Paleveda, H. Schwam, H. Barkemeyer, B. H. Arison, D. F. Veber, R. G. Denkewalter, and R. Hirschmann, *J. Am. Chem. Soc.*, **90**, 3254 (1968).

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(3) E. Schröder and Lubke, "The Peptides," translated by E. Gross, Vol. 1, Academic Press, New York, N. Y., 1965, pp 236–239.

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(5) R. G. Denkewalter, H. Schwam, R. G. Strachan, T. E. Beesley, D. F. Veber, E. F. Schoenewaldt, H. Barkemeyer, W. J. Paleveda, T. A. Jacob, and R. Hirschmann, *J. Amer. Chem. Soc.*, **88**, 3163 (1966).

2.64 mmol of V of 0.42 mmol of III was air oxidized at pH 6.5, the resulting solution was shown by thin layer chromatography to contain VI, VII, and I. Separation of the mixture on Sephadex G-25 led to the elution of the three components in the expected order (*i.e.*, VI, then I, then VII). The yield of I (83% based on III) was consistent with statistical control of the reaction. I was crystallized from water–ethanol and gave a good amino acid analysis after acid hydrolysis. Elemental analysis as well as equivalent weight determination indicated the presence of 1.5 equiv of water of hydration. As would be expected, I showed three titratable protons on base titration, one of $pH_{1/2}$ 4.5 and the other two of average $pH_{1/2}$ 7.5.

The oxidation of a mixture of cysteine peptides has only rarely been applied to the synthesis of unsymmetrical cystine peptides. It is, however, a practical one in a case such as this, where the two peptides and the resulting three products are readily separated.

Biology.[8]—The peptide I has been shown to be devoid of any insulin-like activity in a variety of *in vivo* and *in vitro* insulin assays, for example, in fat cell (glucose utilization and CO_2 production),[9] fat pad, diaphragm, and diabetic rat assays.[10]

Experimental Section

L-Leucyl-L-valyl-S-benzyl-L-cysteinylglycyl-L-glutamic Acid (II).—Glutamic acid (3.675 g, 0.025 mol) was dissolved in 250 ml of 1 *M* potassium borate buffer at pH 9.5. The solution was cooled to 0° and 2.954 g (0.0253 mol) of 2,5-thiazolidinedione was added with vigorous stirring and the pH of the solution was held at 9.5 by the addition of 6 *N* KOH. When base consumption ceased (about 10 min) the pH was adjusted to 2.5 with concentrated H_2SO_4 and the solution was purged of COS with nitrogen (1 hr). Thin layer chromatography showed no detectable glutamic acid, a trace of glycine, and a single new major component, Gly–Glu. The solution was placed in an ice-jacketed Waring Blendor, cooled to 0°, and the pH was adjusted to 10.2 with 50% KOH. S-Benzyl-L-cysteine N-carboxyanhydride (6.26 g, 0.0264 mol) was then added with high-speed blending and pH of the solution was maintained at 10.2 by the addition of 6 *N* KOH until base consumption ceased. The pH was then adjusted to 3.0 by addition of concentrated H_2SO_4, and the solution was filtered and purged of CO_2 with a stream of N_2 (15 min). A 10-ml aliquot of the reaction solution was removed and the remaining solution was cooled to 0°; the pH was again adjusted to pH 10.2 and L-valine N-carboxyanhydride (3.77 g, 0.0264 mol) was added with high-speed blending in the usual manner. The pH was then adjusted to pH 3.0 and the CO_2 was removed as above. The solution was again cooled to 0°, the pH was adjusted to 10.2, L-Leucine N-carboxyanhydride (4.334 g, 0.0276 mol) was added with high-speed blending and the pH was maintained at 10.2 until base consumption ceased. The solution was filtered through Celite and after adjusting the pH to 7.0 with concentrated H_2SO_4, it was filtered again. The pH of the resulting solution was adjusted to pH 3.6, whereupon II (contaminated with traces of other peptides) precipitated. The product was isolated by filtration with no attempt made to isolate the amount of II which remained in solution. The isolated solid (5.4 g) was dissolved in 125 ml of H_2O at pH 6.6 and crystallized by adjusting the pH to 3.6. This material (4.5 g) was recrystallized in the same manner, and the product was isolated by filtration and dried *in vacuo* to give 3.98 g (27%) of II which showed a single ninhydrin-positive component on thin layer chromatography (silica gel H, butanol–acetic acid–water, 10:2.3:6). After acid hydrolysis an amino acid analysis gave $Leu_{0.99}Val_{0.95}Cys(Bzl)_{1.0}Gly_{1.01}Glu_{1.04}$.

L-Leucyl-L-valyl-L-cysteinylglycyl-L-glutamic Acid (III).—L-Leu-L-Val-L-Cys(Bzl)-Gly-L-Glu (3.6, 6 mmol) was dissolved in 100 ml of liquid ammonia. Sodium was added in small portions until the blue color persisted for 3 min. After the addition of 0.5 g of ammonium chloride, the ammonia was removed in a stream of nitrogen and the residual solid was dried *in vacuo*. This material was dissolved in 60 ml of water and the pH was adjusted to 6.5 with acetic acid. Based on the amounts of I and VI which were isolated from an aliquot of this solution (see below), the concentration of III was 0.042 mmol/cc or a 42% yield for the reduction. Air was bubbled through a 50-ml aliquot of this solution until it no longer reduced nitroprusside. The pH of the solution was brought to 3.8 with acetic acid and the solution evaporated to dryness. After dissolution in 10 ml of water and refrigeration overnight, a white precipitate of the disulfide VI was formed. Filtration gave 0.853 g (33% based on II), dec pt 232°, $[\alpha]^{25}D$ −56° (1% in 50% aqueous acetic acid). After acid hydrolysis an amino acid analysis gave $Gly_{1.02}Glu_{1.02}Val_{0.96}Leu_{1.00}\frac{1}{2}Cys_{\sim 1}$. *Anal.* Calcd for $C_{42}H_{72}N_{10}O_{16}S_2 \cdot 4H_2O$: C, 45.48; H, 7.27; N, 12.63; S, 5.78; wt loss on drying, 6.5. Found: C, 45.54; H, 7.13; N, 12.57; S, 5.82; wt loss on drying, 6.2.

S-Benzyl-L-cysteinyl-L-asparagine (IV).—Asparagine (6.605 g, 0.05 mol) was dissolved in 200 ml of 1 *M* potassium borate buffer, the solution was cooled to 0° in an ice-jacketed Waring Blendor, and the pH was adjusted to 10.2 with 50% KOH. To this was added 11.86 g (0.05 *M*) of S-benzyl-L-cysteine N-carboxyanhydride with high-speed stirring. The pH was maintained at 10.2 throughout the reaction (11 min) by the addition of 50% KOH. When base consumption ceased, the solution was filtered, the pH was adjusted to 4.5 with concentrated H_2SO_4, and the solution was freeze dried. The resulting solid was extracted with methanol until subsequent extracts no longer gave a positive ninhydrin test. The combined extracts were evaporated to dryness *in vacuo* and crystallized from water–ethanol to give 9.09 g (56%) of IV. Recrystallization from water (83% recovery) gave an analytical sample, $[\alpha]^{25}D$ 21.4° (1% in 50% acetic acid). *Anal.* Calcd for $C_{14}H_{19}N_3O_4S$: C, 51.68; H, 5.92; N, 12.91; S, 9.86; equiv wt, 325. Found: C, 51.58; H, 5.72; N, 13.01; S, 9.87; equiv wt, 330 (base titration, $pH_{1/2}$ 6.7).

L-Cysteinyl-L-asparagine (V).—S-Benzyl-L-cysteinyl-L-asparagine (1.30 g, 4 mmol) was reduced with sodium in liquid ammonia as described for III. The crude solid product was dissolved in 40 ml of water and the pH was brought to 6.5 with acetic acid. Based on the amounts of I and VII which were isolated from an aliquot of this solution (see below), the concentration of V was 0.08 mmol/cc or an 88% yield for the reduction. Air was bubbled through a 10-ml aliquot of this solution until it no longer reduced nitroprusside reagent. This solution was used to identify VII on tlc. A sample of VII isolated in the preparation of I (below) showed, after hydrolysis with aminopeptidase M, $\frac{1}{2}Cys_{1.0}Asn_{0.9}$.

L-Leucyl-L-valyl-S-(L-cysteinyl-L-asparagine)-L-cysteinyl-glycol-L-glutamic Acid (I).—The solution of III (10 ml, 0.42 mmol) prepared as described above was mixed with 30 ml (2.64 mmol) of the solution of V prepared as described above. Air was bubbled through this solution until it no longer reduced nitroprusside reagent. Thin layer chromatography showed the presence of I, VI, and VII. The solution was concentrated to a volume of 6-ml and put on a 100 × 2.5 cm Sephadex G-25 column. The column was developed with water and 8-ml fractions were collected. Cuts 36–40 contained VI (33 mg, 17% yield based on III). Cuts 42–47 contained I (268 mg, 83% yield based on III, 13% based on V). Cuts 49–54 contained VII (597 mg, 87% yield based on V). The fractions containing I were combined and crystallized from water–ethanol to give 105 mg of I, mp 203–206° dec, $[\alpha]^{25}D$ −8.0° (0.49% in 1% aqueous acetic acid). *Anal.* Calcd for $C_{28}H_{48}N_8O_{12}S_2 \cdot 1.5H_2O$: C, 43.12; H, 6.59; N, 14.37; equiv wt, 260. Found: C, 43.06; H, 6.62; N, 14.39; equiv wt, 259 (base titration). After acid hydrolysis an amino acid analysis gave $Asp_{1.00}Gly_{1.02}Glu_{1.02}Leu_{0.98}Val_{0.98}\frac{1}{2}Cys_{\sim 2}$.

(6) R. Hirschmann, R. G. Strachan, H. Schwam, E. F. Schoenewaldt, H. Joshua, H. Barkemeyer, D. F. Veber, W. J. Paleveda, T. A. Jacob, T. E. Beesley, and R. G. Denkewalter, *J. Org. Chem.*, **32**, 3415 (1967).

(7) III has been previously reported as a crude product and was not characterized: G. L. Tritsch and D. W. Woolley, *J. Amer. Chem. Soc.*, **82**, 2787 (1960).

(8) We are greatly indebted to Drs. H. Katzen and M. Glitzer and Mr. J. Humes for permission to include these biological results.

(9) M. Rodbell, *J. Biol. Chem.*, **239**, 375 (1964).

(10) G. A. Stewart, *Brit. Med. Bull.*, **16**, 196 (1960), and references cited therein.

Registry No.—I, 18872-79-2; II, 18872-80-5; IV, 18872-88-3; VI, 18872-87-2.

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Phosphonoacetaldehyde

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De Koning[3] has reported that the reaction of 2-aminoethylphosphonic acid (**1**, 2-AEP) with ninhydrin produces acetaldehyde and inorganic phosphate as a result of oxidation, followed by cleavage of the C–P bond. It was assumed that phosphonoacetaldehyde (**2**) was first formed when 2-AEP was heated with ninhydrin at 96° and at pH 5 for 8 hr. Attempts to

$$\underset{\mathbf{1}}{H_2NCH_2CH_2PO(OH)_2} \xrightarrow{\text{ninhydrin}} [\underset{\mathbf{2}}{OCHCH_2PO(OH)_2}] \xrightarrow[\text{pH 5}]{96^\circ} CH_3CHO + PO_4^{3-}$$

isolate and characterize phosphonoacetaldehyde (**2**) were not mentioned.

The decomposition of **2** is rather surprising because the C–P bond is usually stable with respect to hydrolytic cleavage, even under vigorous reaction conditions.[4] The normal procedure for the hydrolysis of a phosphonate ester is to heat the ester under reflux with 6 *N* hydrochloric acid for 48 hr or longer; normally no detectable C–P cleavage takes place. However, Chavane has stated that the polarity of the substituents on the alkyl chain has a large influence on the stability of phosphonic acids and predicted that **2** would be unstable.

We first tried to convert diethyl phosphonoacetaldehyde diethyl acetal[5] (**3**) to **2**. The problem was to remove the ester groups without destroying other portions of the molecule. One of the methods tried involved heating **3** with excess thionyl chloride in the hope of converting the diester to the phosphonyl dichloride. Instead, a thermally unstable compound was recovered, which proved to be monoethyl 2-ethoxyvinylphosphonochloridate (**4**) (Scheme I). The

SCHEME I

$$\underset{\mathbf{3}}{(C_2H_5O)_2CHCH_2PO(OC_2H_5)_2} \xrightarrow{SOCl_2} \underset{\mathbf{4}}{C_2H_5OCH{=}CHPO(OC_2H_5)Cl}$$

$$\downarrow SOCl_2 \qquad \downarrow H_2O$$

$$(C_2H_5O)_2CHCH_2POCl_2 \qquad \underset{\mathbf{5}}{OCHCH_2PO(OC_2H_5)OH}$$

structure of **4** was determined from its ir and its nmr spectra and from its chemical reactions.

This line of research was discontinued in favor of a second route that appeared to offer more promise. Lutsenko and Kirilov[6] synthesized 2-acetoxy-2-chloroethylphosphonyl dichloride (**6**) but for some reason they did not report the hydrolysis of **6** to **2**. The

$$CH_3CO_2CH{=}CH_2 + PCl_5 + SO_2 \longrightarrow \underset{\mathbf{6}}{CH_3CO_2CHClCH_2POCl_2} \xrightarrow{H_2O} \mathbf{2}$$

hydrolysis took place readily in an aqueous tetrahydrofuran solution at room temperature to give a nearly quantitative yield of **2** as a slightly yellow, viscous oil. This crude oil was shown to be at least 81% **2** by converting it to the crystalline 2,4-dinitrophenylhydrazone of **2**.

Oxidation of **2** with aqueous, alkaline permanganate gave phosphonoacetic acid in 68% yield, identical with the acid produced by the hydrolysis of triethyl phosphonoacetate. We have converted diethyl phosphonoacetaldehyde (**7**) to the biologically important 2-AEP (**1**) by the sequence shown in Scheme II. Phono-

SCHEME II

$$\underset{\mathbf{7}}{OCHCH_2PO(OC_2H_5)_2} \longrightarrow [HON{=}CHCH_2PO(OC_2H_5)_2] \longrightarrow$$

$$[CH_3CONHCH_2CH_2PO(OC_2H_5)_2] \longrightarrow \underset{\mathbf{1}}{H_2NCH_2CH_2PO(OH)_2}$$

acetaldehyde (**2**) has recently been shown to be a normal constituent of at least one biological system and there is good evidence that it is an enzymic breakdown product of **1**.[7]

The authentic **2** also provided the opportunity to determine its stability under the conditions reported by De Koning.[3] Heating a solution of **2** in aqueous acetate buffer (pH 5) for 8 hr at 90° produced good yields of acetaldehyde, isolated as the 2,4-dinitrophenylhydrazone, and of phosphate, recovered as the $MgNH_4$ salt. Thus, even though phosphonoacetic acid is stable to prolonged heating with 6 *N* hydrochloric acid, **2** contains a C–P bond which hydrolyzes at a moderate rate under much milder conditions, even though it appears to have good stability in aqueous solutions at low and at high pH values at 25°.

Experimental Section

Melting points were determined with a Hershberg[8] melting point apparatus and are corrected; boiling points are uncorrected. Analyses were performed by Galbraith Laboratories, Inc., Knoxville, Tenn., except for the Cl analysis of **4** which was determined by adding a sample of **4** to water, followed by HNO_3

(1) To whom inquiries should be addressed.
(2) Taken in part from the Ph.D. dissertation of L. F. E., 1968.
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(8) E. B. Hershberg, *Ind. Eng. Chem., Anal. Ed.*, **8**, 312 (1936).