mentation constant, crystallizability, biological activity, ratio of maximum/minimum absorptivities in the ultraviolet, infrared absorption spectra and total nitrogen content. Of great interest, also, was the observation that some sixty molecules of water per molecule of insulin (mol. wt. 12,000) seem so tightly bound that they do not react with the solvent.[2] At the low temperatures at which the experiments were carried out, these water molecules (amounting to approximately 9 g. per 100 g. of insulin) might be "frozen" into or onto the protein hydrogen bond network, or might be arranged in a high state of order around CH_2 or CH_3 groups, as observed in some cases.[3] This observation would lead to the conclusion that water molecules play an important part in the stabilization of protein structure whether in solution or in the crystalline state, since a similar role on the part of water molecules is also inferred from studies on small peptides composed of amino acid residues having like configuration.[4]

Experimental

Insulin.—Three different samples of insulin were studied. Two samples were crystalline beef zinc insulin, kindly furnished through the courtesy of the Eli Lilly Co., lots 296,000 and T-2344 (5 times recrystallized). The third sample was amorphous, zinc free, isoelectrically precipitated insulin prepared from crystalline zinc insulin, kindly furnished us by the Armour Co. The biological activities were 25, 27 and 27 I.U./mg., respectively. Extensive physical chemical studies were carried out on lot 296,000[5a,b] and on lot T-2344.[6] Each preparation was thoroughly equilibrated at room temperature until the moisture content, as determined by drying *in vacuo* at 56° over phosphorus pentoxide, became constant. Samples equilibrated in this manner contained 9.3 ± 0.5% moisture.

Liquid Ammonia Studies.—A three-neck flask was equipped with a vacuum tight stirrer in the center, a soda lime–calcium chloride drying tube at one end, and a gas inlet tube at the other. The gas inlet tube was connected to a three-way stopcock, one end of which was connected to a tank of purified, oil pumped nitrogen, and the other through a spiral glass tube to a tank of ammonia. The apparatus was flushed with dry nitrogen at room temperature for one-half hour, and then placed into a Dewar flask kept at −70°. Flushing with dry nitrogen was continued for another 20 minutes. The flask was removed from the cold bath, wiped dry on the outside, and examined for evidence of condensation. Should moisture have condensed on the inside of the apparatus, it was disconnected and another assembly set up. Absence of moisture is critical, since it was observed that slight traces of water will form ammonium hydroxide which will subsequently produce an insoluble precipitate of denatured insulin.

If no condensation was observed, the assembly was returned to the cold bath, and ammonia introduced. This gas will condense faster if it is precooled by placing the spiral glass tube into a bath kept at −33°. When 50 ml. of liquid ammonia was collected, nitrogen was admitted, and the flask transferred to a bath kept at −36°. With nitrogen constantly passing over the surface of the solution, the drying tube was quickly lifted and some 200 mg. of insulin poured into the flask from an inverted test-tube. The drying tube was replaced, the stirrer turned on for a few minutes, and the solution kept at −36° for 18 hours. Under these conditions all of the insulin goes into solution almost as soon as it comes in contact with the liquid ammonia, resulting in a pale yellow solution free of any precipitate.

At the end of the experiment the flask was removed from the cold bath and ammonia removed by means of a stream of nitrogen. The last traces of ammonia were removed by placing the flask in an oil-bath at 60° for two hours and then connecting the flask at room temperature to a vacuum pump without traps for one-half hour, breaking the vacuum by means of dry nitrogen admitted through a drying tube. A small sample of insulin was quickly removed from the flask to a small container previously dried to constant weight, a weight was quickly taken and the sample dried over phosphorus pentoxide at 56° *in vacuo*. Under these conditions, the moisture content of the liquid ammonia-treated insulin samples was found to be 9.0 ± 0.7%. Total nitrogen, based on dry insulin, was identical with values obtained on the starting material,[5b,6] indicating that ammonia was not adsorbed on the molecule, nor that it had reacted with any functional group. The ultraviolet absorption spectra at *p*H 2.2 and 7.4 were identical with those of the starting material,[5b] as were solubility and behavior in the analytical ultracentrifuge as function of charge and ionic strength. Insulin treated with liquid ammonia could be recrystallized either as the zinc salt or as the acid sulfate. The biological activities of the liquid ammonia-treated samples without further purification were 24 ± 2 I.U./mg.[7]

Infrared Spectra.—Infrared spectra were determined as previously described.[8] Fluorolube mulls were used above 1380 cm.$^{-1}$, and nujol mulls between 1380 and 625 cm.$^{-1}$. Since the infrared spectra of the three starting materials were identical with those of the three liquid ammonia treated samples, one spectrum only is shown in Fig. 1 as representative for the whole group. Bands are located at 3230 and 3030 (NH stretch), 2940 (CH stretch), 1700 (hydrogen bonded CO stretch), 1636, 1538 (CO, CN stretch due to resonating peptide linkage), 1500, 1268 (CH stretch and/or NH deformation from peptide linkage), 1463 (symmetrical COO^-), 1444 (CH deformation), 1003 (CNC stretch), 976, 918 (side chain rocking, CH rocking), and 1392, 1237, 1174, 1120, 836 cm.$^{-1}$ (unassigned).[4]

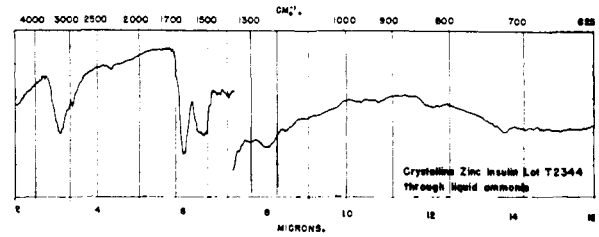

Fig. 1.—Infrared absorption spectrum of liquid ammonia-treated crystalline zinc insulin, Lot T2344.

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(2) It is planned to explore this observation further by studies employing deuterium oxide aud water containing O-18.

(3) H. Frank, personal communication.

(4) E. Ellenbogen, THIS JOURNAL, **78**, in press.

(5) (a) J. L. Oncley and E. Ellenbogen, *J. Phys. Chem.*, **56**, 85 (1952); (b) E. Ellenbogen, Ph.D. Thesis, Harvard University, 1949.

(6) E. Fredericq and H. Neurath, THIS JOURNAL, **72**, 2684 (1950).

(7) We are indebted to the Eli Lilly Co. for some of these assays.

(8) The aid of Dr. Foil A. Miller, Mellon Institute, Pittsburgh, who determined these spectra, is gratefully acknowledged.

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The Preparation of Amides of Arylphosphonic Acids. III. Amides of Secondary Amines[1]

BY LEON D. FREEDMAN AND G. O. DOAK

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It long has been known that arylphosphonic dichlorides react with a wide variety of primary

(1) Presented, in part, before the Division of Organic Chemistry at the 126th Meeting of the American Chemical Society, New York, N. Y., September, 1954.

TABLE I

ARYLPHOSPHONIC DIAMIDES OF SECONDARY AMINES

Compound	Yield, %	M.p.,[a] °C.	Formula	Phosphorus,[b] % Calcd.	Phosphorus,[b] % Found	Nitrogen,[c] % Calcd.	Nitrogen,[c] % Found
R = P-(*p*-Nitrophenyl)-phosphonic							
R diaziridide	90	107–110	$C_{10}H_{12}N_3O_3P$	12.23	11.92	16.60	16.52
R dipyrrolidide[d,e]	74	67.5–69.5	$C_{14}H_{20}N_3O_3P$	10.02	9.85	13.59	13.52
R dipiperidide[d,f]	71	139–142	$C_{16}H_{24}N_3O_3P$	9.18	9.14	12.46	12.31
R morpholide[d,g]	68	204–206.5	$C_{14}H_{20}N_3O_5P$	9.08	9.06	12.31	12.31
N,N'-Tetraethyl-R diamide[h,i]	39	88–92	$C_{14}H_{24}N_3O_3P$	9.89	9.74	13.41	13.34
R = P-(*p*-Aminophenyl)-phosphonic							
R dipyrrolidide[f,j]	65	191–193	$C_{14}H_{22}N_3OP$	11.09	10.87	15.05	14.97
R dipiperidide[f,j]	84	198–201	$C_{16}H_{26}N_3OP$	10.08	10.01	13.67	13.58

[a] Melting points were taken as previously described; *cf.* G. O. Doak and L. D. Freedman, THIS JOURNAL, **73**, 5658 (1951). [b] Phosphorus was determined by the method of B. C. Stanley, S. H. Vannier, L. D. Freedman and G. O. Doak, *Anal. Chem.*, **27**, 474 (1955). [c] Nitrogen was determined by a micro-Kjeldahl procedure. [d] The solvent used for the condensation was carbon tetrachloride. [e] The crude diamide was purified by dissolution in ether, treatment of this solution with charcoal, and evaporation of the clarified solution to dryness. [f] Recrystallized from aqueous ethanol. [g] The crude diamide was washed first with carbon tetrachloride, and then with water until the washings were free of chloride ion. The residue after drying in a desiccator proved to be the pure dimorpholide. [h] The solvent used for the condensation was acetone. [i] Recrystallized from ether. [j] The solvent used in the reduction was absolute ethanol.

TABLE II

PHOSPHONAMIDIC ANHYDRIDES

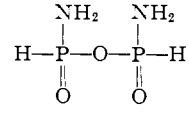
$[p\text{-}O_2NC_6H_4PO(NR_2)]_2O$ R_2NH =	Yield, %	M.p., °C.	Formula	Phosphorus, % Calcd.	Phosphorus, % Found	Nitrogen, % Calcd.	Nitrogen, % Found
Diethylamine[a]	6–19[b] 0–30[c]	116.5–118.5	$C_{20}H_{28}N_4O_7P_2$	12.43	12.17	11.24	11.25
Dipropylamine[d]	0–24[c]	77–80	$C_{24}H_{36}N_4O_7P_2$	11.17	11.06	10.10	9.98
Diisopropylamine[e]	0–44[c]	>200[f]	$C_{25}H_{36}N_4O_7P_2$	11.17	11.02	10.10	10.11
Diisobutylamine	0–64[c]	125–128.5	$C_{28}H_{44}N_4O_7P_2$	10.15	10.07	9.18	9.19
2-Methylpiperidine[g]	29,[h] 33[h]	170.5–173.5	$C_{24}H_{32}N_4O_7P_2$	11.26	11.09	10.18	10.11

[a] Calcd.: C, 48.19; H, 5.66; mol. wt., 498.4. Found: C, 48.43; H, 5.83; mol. wt., 506.5 (in benzene by lowering of the freezing point). [b] This range of yields was obtained when the acid chloride was dissolved in carbon tetrachloride and the amine was dissolved in benzene. [c] This range of yields was obtained when acetone was used as the solvent. [d] Calcd.: C, 51.98; H, 6.54. Found: C, 51.55; H, 6.57. [e] Calcd.: C, 51.98; H, 6.54. Found: C, 52.25; H, 6.57. [f] M.p. varied greatly with the rate of heating. [g] Calcd.: C, 52.36; H, 5.86; mol. wt., 550.5. Found: C, 52.47; H, 5.89; mol. wt., 536.5 (in benzene by lowering of the freezing point). [h] This yield was obtained when carbon tetrachloride was used as the solvent.

amines to form arylphosphonic diamides.[2] On the other hand, the chemical literature[2] records only four arylphosphonic diamides derived from secondary amines; moreover, the only secondary amines used were piperidine and tetrahydroquinoline. A study of the reaction of phosphonic dichlorides with secondary amines was begun in this Laboratory about two years ago. The problem proved to be much more difficult than in the case of the primary amines. In spite of considerable work the results obtained are fragmentary and difficult to interpret. We are reporting them at this time since this investigation has been indefinitely interrupted.

When *p*-nitrophenylphosphonic dichloride was condensed with ethylenimine, pyrrolidine, piperidine or morpholine by procedure 1 as previously described,[3] the expected diamides were consistently obtained without difficulty. Two of the nitro-substituted diamides were reduced to the corresponding amino derivatives with Raney nickel and hydrogen at 40 lb. pressure. The analyses, yields and m.p.'s of these compounds are listed in Table I.

The reaction between *p*-nitrophenylphosphonic dichloride in carbon tetrachloride and diethylamine in benzene produced approximately 2 moles of amine hydrochloride per mole of phosphonic dichloride used. However, we were unable to isolate any of the expected diamide from the reaction mixture. Instead we obtained a neutral substance in which the nitrogen to phosphorus ratio was only 2:1. Therefore, this substance cannot be a diamide. Since the substance is non-acidic, it cannot be either a monoamide or an amine salt of the phosphonic acid. The analysis and molecular weight of the material suggest that it is a derivative of the hypothetical compound, phosphonamidic anhydride



Compounds of the same type were also obtained with dipropylamine, diisopropylamine, diisobutylamine and 2-methylpiperidine. The phosphonamidic anhydrides prepared together with their analyses, yields and m.p.'s are given in Table II.

Since the phosphonamidic anhydrides were prepared under reaction conditions in which no water was added, it is rather surprising that we isolated compounds, the structure of which demands that a mole of water be furnished for each mole of product formed. It should be emphasized that these reactions were run under "anhydrous conditions," *i.e.*, the reactants and the solvent were stored over Drierite, and the reaction vessel was equipped

(2) *Cf.* G. M. Kosolapoff, "Organophosphorus Compounds," John Wiley and Sons, Inc., New York, N. Y., 1950, pp. 316–317.

(3) G. O. Doak and L. D. Freedman, THIS JOURNAL, **76**, 1621 (1954).

with a sealed stirrer, a special dropping funnel (Ace Glass Cat. No. 7347), and a condenser with an attached calcium chloride tube. However, the reactants were exposed for a short time to the laboratory atmosphere when they were transferred to the reaction vessel. Moreover, the amine hydrochloride formed in the reaction was removed by filtration without any attempt to prevent the ingress of moisture. It appears that the phosphonamidic anhydrides must be formed by traces of water inadvertently introduced from the laboratory atmosphere. The following reaction sequence is suggested

$$p\text{-}O_2NC_6H_4POCl_2 + 2R_2NH \longrightarrow p\text{-}O_2NC_6H_4PO(NR_2)Cl + R_2NH_2Cl \quad (1)$$

$$2\ p\text{-}O_2NC_6H_4PO(NR_2)Cl + H_2O + 2R_2NH \longrightarrow [p\text{-}O_2NC_6H_4PO(NR_2)]_2O + 2R_2NH_2Cl \quad (2)$$

This hypothesis is consistent with the fact that we have obtained widely varying yields of anhydride in duplicate runs. However, the deliberate introduction of water (0.5 mole per mole of phosphonic dichloride) during the condensation has given us materials which we have been unable to purify. The addition of water 0.5 hour or 20 hours after the reaction mixture had been refluxed failed to give larger yields of phosphonamidic anhydrides. With diethylamine and diisopropylamine the reaction has been studied under a variety of experimental conditions. With diethylamine a total of 16 and with diisopropylamine a total of 24 reactions were run. We have not found reaction conditions whereby the phosphonamidic anhydrides can be prepared in reproducible yields.

When *p*-nitrophenylphosphonic dichloride was condensed with diethylamine in acetone, we did succeed in obtaining the corresponding diamide in two experiments (*cf.* Table I). However, in other experiments in which acetone was used, we isolated only the phosphonamidic anhydride. Further work obviously is required to determine why different results are obtained under apparently identical reaction conditions. The reaction between *p*-nitrophenylphosphonic dichloride and dipropylamine, diisopropylamine, diisobutylamine or 2-methylpiperidine yielded phosphonamidic anhydrides; no diamide was ever isolated from these reactions.

Experimental

The preparation of *p*-nitrophenylphosphonic dichloride has been described previously.[3] Diisopropylamine was kindly furnished by Carbide and Carbon Chemicals Co. Ethylenimine was purchased from the Chemirad Corporation. The other amines were obtained from the Eastman Kodak Co. With the exception of ethylenimine, all the amines were dried over Drierite and fractionated before use. The solvents used in the condensations were also dried over Drierite and fractionated.

The Condensation of *p*-Nitrophenylphosphonic Dichloride with Secondary Amines.—The method used for preparing both the diamides and the phosphonamidic anhydrides was similar to procedure 1 as previously described.[3] An example of the preparation of a phosphonamidic anhydride (N,N-diethyl-P-(*p*-nitrophenyl)-phosphonamidic anhydride) is given below. The synthesis of P-(*p*-nitrophenyl)-phosphonic diaziridide and N,N-diisopropyl-P-(*p*-nitrophenyl)-phosphonamidic anhydride differs somewhat from the general procedure; the preparation of these two compounds is described below in detail.

P-(*p*-Nitrophenyl)-phosphonic Diaziridide.—A solution of 4.70 g. of *p*-nitrophenylphosphonic dichloride in 150 ml. of carbon tetrachloride was added to an ice-cold solution of 4.5 g. (excess) of anhydrous ethylenimine in 50 ml. of carbon tetrachloride. When all the acid chloride had been added, the mixture was allowed to stand overnight at room temperature. A white precipitate was removed by filtration, washed with carbon tetrachloride and discarded. When the filtrate and washings were concentrated *in vacuo* to about 10 ml., crystals were obtained which were washed with about 10 ml. of ether. These crystals after drying in a desiccator gave satisfactory analytical values for the desired diamide.

N,N-Diethyl-P-(*p*-nitrophenyl)-phosphonamidic Anhydride.—To a stirred solution of 8.9 ml. of diethylamine in 25 ml. of acetone was added 5.18 g. of *p*-nitrophenylphosphonic dichloride dissolved in 100 ml. of acetone. The mixture was refluxed gently for 1 hour and then allowed to stand overnight at room temperature. The diethylamine hydrochloride was removed by filtration and washed with acetone. The combined filtrate and washings were evaporated to dryness (either on the steam-bath or at room temperature), and the residue was recrystallized from aqueous alcohol.

N,N-Diisopropyl-P-(*p*-nitrophenyl)-phosphonamidic Anhydride.—This compound was prepared by a procedure similar to that used for the other phosphonamidic anhydrides. Acetone was used as the solvent for the condensation. (No phosphonamidic anhydride was obtained when carbon tetrachloride was used as the solvent.) After the reaction mixture was allowed to stand overnight, the precipitate was removed by filtration and washed with water until the washings were free of chloride ion. The residue consisted of the analytically pure phosphonamidic anhydride. A second crop was obtained by evaporating the acetone mother liquors to dryness and recrystallizing the residue from 95% ethanol.

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Quinoxaline Studies. VII. A Quinoxaline Analog of Pteroic Acid

BY JOHN DRUMHELLER[1] AND HARRY P. SCHULTZ

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Martin, *et al.*,[2] found N-(4-[(4-quinazolyl)-amino]-benzoyl)-glutamic acid to be a microbiological growth factor with a potency of from 0.01 to 0.1 that of pteroylglutamic acid. More recently Leese and Rydon[3] reported the synthesis of two quinoxaline analogs of pteroic acid, 4-[2-quinoxalyl)-methylamino]-benzoic acid and 4-[(3-hydroxy-2-quinoxalyl)-methylamino]-benzoic acid. The purpose of this note is to report the synthesis of 4-[(2-quinoxalyl)-amino]-benzoic acid.

2-Hydroxyquinoxaline was prepared by the methods of Goldweber and Schultz[4] as well as Gowenlock, *et al.*[5] This was transformed into 2-chloroquinoxaline according to the procedure of Gowenlock, *et al.*[5] Attempts to prepare 2-chloroquinoxaline in one step by direct reaction between

(1) Abstracted from the M.S. thesis of John Drumheller, The University of Miami.

(2) G. J. Martin, J. Moss and S. Avakian, *J. Biol. Chem.*, **167**, 737 (1947).

(3) C. L. Leese and H. N. Rydon, *J. Chem. Soc.*, 308 (1955).

(4) M. Goldweber and H. Schultz, THIS JOURNAL, **76**, 287 (1954).

(5) A. H. Gowenlock, G. T. Newbold and F. S. Spring, *J. Chem. Soc.*, 622 (1945).