variables, will be required before a generally consistent account can be given.

Experimental[17,18]

Substrates.—D-Phenylalanine was obtained in a 60% yield and L-phenylalanine in a 73% yield from DL-phenylalanine (Dow) by conversion of acetyl-DL-phenylalanine into acetyl-L-phenylalanine-*p*-toluidide with the aid of cysteine-activated papain and subsequent hydrolysis of the above toluidide and the residual acetyl-D-phenylalanine to the corresponding amino acids.[19] The acylated D- and L-phenylalanines used in this study were prepared as described in Table III and their properties are given in Table IV. The phenylhydrazine hydrochloride was a recrystallized product.

Enzyme Experiments.—The experiments summarized in Table I were conducted in the following manner. The various acylated phenylalanines were weighed into stoppered flasks, the indicated amounts of phenylhydrazine hydrochloride and L-cysteine hydrochloride added, sufficient 0.5 *M* acetic acid–0.5 *M* sodium acetate buffer added at 40° to effect complete solution of the solids in each flask, the *p*H of each solution adjusted to 4.60 by the addition of 4.5 *N* aqueous sodium hydroxide, the required amount of enzyme solution[20] added, the solution adjusted to volume with the buffer, and immediately incubated at 40°. All experiments reported in Table I were started simultaneously. At the stated time intervals (*cf.* Table I) the precipitates in each flask were collected, on sintered glass filters, washed with water, dried and weighed. The precipitates were then extracted with boiling ethanol until no significant amount of ethanol-soluble material remained on the filters and the loss in weight upon ethanol extraction taken as the yield of phenylhydrazide. The ethanol extracts were concentrated and the phenylhydrazides recovered, the properties of which are given in Table II. From the precipitates collected after the fourth day of incubation an ethanol insoluble substance was recovered which was identified as L-cystine, both by decomposition point and specific rotation. After 24 days incubation at 40° and after all insoluble fractions had been collected the solutions were acidified with aqueous hydrochloric acid and the residual acylated phenylalanines recovered *via* a continuous liquid-liquid ether extraction. The results of these operations are summarized in Table V.

TABLE V

ISOLATION OF UNREACTED SUBSTRATES

Phenylalanine	Quantity (g.) Present	Isolated	Recovery, %	M. p., °C. Crude	Recryst.[a]	Orig.[b]
CH_3CO-D-[c]	0.35	0.28	80	163–166	167–168	170–171
C_6H_5CO-D-	.27	.23	91[d]	134–136	142	142–143
$C_6H_5SO_2$-D-	.71	.66	93	123–126	133	133
C_6H_5NHCO-L-	.57	.45	79	159–163	166–167	171
C_6H_5NHCO-D-	.57	.48	84	159–161	166–167	171

[a] One recrystallization from solvents indicated in Table III. [b] *Cf.* Table IV. [c] From experiment with an enzyme concentration of 25 g. per liter. [d] Corrected for the 9% yield of phenylhydrazide isolated.

Summary

The papain-catalyzed synthesis of phenylhydrazides of carboethoxy- and carbobenzoxy-D-phenylalanine has been confirmed and it has been established that under the same conditions the above reaction does not proceed with acetyl-D-phenylalanine. Benzoyl-D-phenylalanine was shown to react to a limited extent.

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(17) The authors wish to express their indebtedness to Dr. A. Elek for all microanalyses reported in this communication.

(18) All melting points are corrected.

(19) The details of this procedure will be described shortly in another communication from these laboratories.

(20) The enzyme solution was prepared by dissolving sufficient freshly purified papain[4] in 0.5 *M* acetic acid–0.5 *M* sodium acetate buffer to give an enzyme concentration of 0.1 g. per ml.

[CONTRIBUTION NO. 1237 FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

A Reinvestigation of the Kinetics of the Urease–Catalyzed Hydrolysis of Urea. I. The Activity of Urease in the Presence of Sodium and Potassium Phosphate

BY GERALD D. FASMAN AND CARL NIEMANN[1]

In an investigation of the factors operative in the urease-catalyzed hydrolysis of urea in aqueous solutions buffered at *p*H 7.0 with sodium or potassium phosphate it has been found that both of the buffer components participate in the hydrolytic reaction and that the buffer anion apparently functions as an activator and the buffer cation as an inhibitor.

In an earlier communication from these laboratories[2] it was concluded from a study of the urease–catalyzed hydrolysis of urea in the presence of potassium phosphate buffers of *p*H 7.0 that the hydrolytic reaction was competitively inhibited by phosphate ion. It is now known that this conclusion is incorrect and that an error was made in assuming that potassium ion was incapable of interaction either with the enzyme or the enzyme–substrate complex. It is the purpose of this communication to show that in the urease-catalyzed hydrolysis of urea at 25° and *p*H 7.0 in the presence of a sodium or potassium phosphate buffer, in addition to their action as buffers, phosphate ion apparently functions as an activator and sodium or potassium ion as an inhibitor.

From the data given in Figs. 1 and 2 it will be seen that at equivalent buffer concentrations and with all other factors held constant the activity of urease is greater in a potassium phosphate buffer than in a sodium phosphate buffer. Thus it appears that sodium ion is a more effective inhibitor of the phosphate–urease–urea system than is potassium ion. Furthermore if sodium chloride is added to a system containing urease, urea and sodium phosphate, or potassium chloride to a system containing urease, urea and potassium phosphate a significant diminution in the activity of the urease is observed (*cf.* Fig. 3). It should not be assumed that chloride ion is without effect upon the above system for it will be shown in a subsequent communication that chloride ion, in common with a number of other anions, can function as an activator of urease. However, at equiv-

(1) To whom inquiries regarding this article should be sent.

(2) K. M. Harmon and C. Niemann, *J. Biol. Chem.*, **177**, 601 (1949).

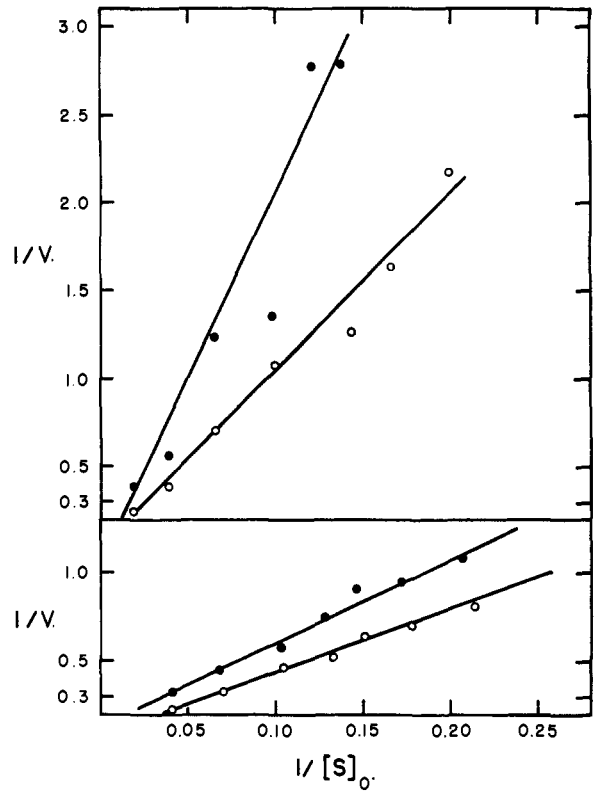

Fig. 1.—Activity of urease in sodium and potassium phosphate buffers: $1/v$ in micromoles of ammonia per ml. per min.; $1/[S]_0$ in micromoles of urea per ml.; solid circles, sodium phosphate; open circles, potassium phosphate; upper plot, 0.055 molar phosphate; lower plot, 0.214 molar phosphate.

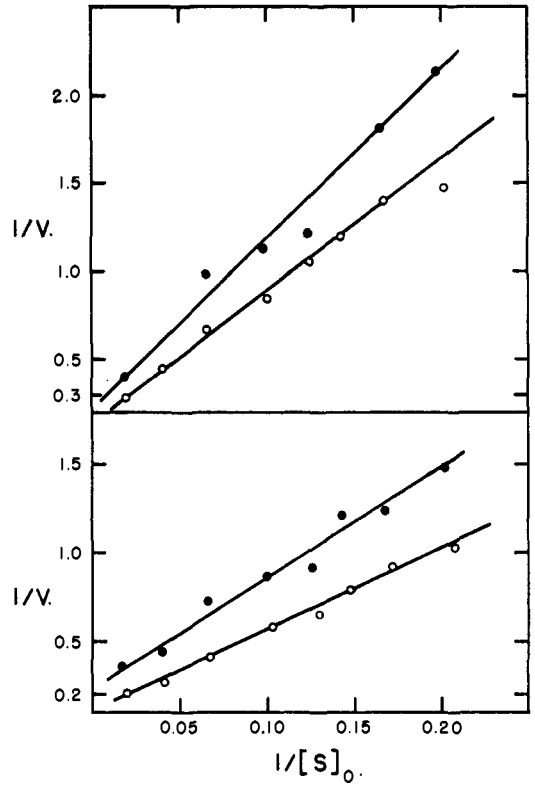

Fig. 2.—Activity of urease in sodium and potassium phosphate buffers: $1/v$ in micromoles of ammonia per ml. per min.; $1/[S]_0$ in micromoles of urea per ml.; solid circles, sodium phosphate; open circles, potassium phosphate; upper plot, 0.267 molar phosphate; lower plot, 0.158 molar phosphate.

alent concentrations and at 25° and *p*H 7.0 chloride ion is much less effective as an activator of urease than is phosphate ion.[3]

With potassium phosphate buffers at 25° and *p*H 7.0 the urease catalyzed hydrolysis of urea appears to follow so-called Michaelis-Menten kinetics in that for any given potassium phosphate concentration the conventional $1/v$ *versus* $1/[S]_0$ plot[4] was found to be linear within the limits of experimental error. However, with increasing potassium phosphate concentration the activity of the urease was first observed to increase and then to decrease (*cf.* Fig. 4) and when the slope of the $1/v$ *versus* $1/[S]_0$ plot for any given potassium phosphate concentration was in turn plotted against potassium phosphate concentration it was noted that the urease exhibited maximum activity at a potassium phosphate concentration of approximately 0.16 *M* for this particular set of experiments (*cf.* Fig. 5). With sodium phosphate buffers the $1/v$ *versus* $1/[S]_0$ plots were not linear except at low sodium phosphate concentrations (*cf.* Fig. 6). However, from the data given in Fig. 6 it is clear that here also the activity of the urease first increased and then decreased with increasing sodium phosphate concentration.

The conclusion that sodium or potassium ion can function as an inhibitor in an activated urease–urea system derives support from earlier observations on the inhibitory action of sodium and potassium chloride in urease–urea systems containing no added buffer.[5,6] In fact if one is prepared to accept the proposition that results of equal validity may be obtained with either crystalline urease or with crude urease preparations it may be said that E. F. and H. E. Armstrong[7] first established the fact that with potassium phosphate buffers the activity of urease is first increased and then decreased with increasing concentrations of potassium phosphate and that at equivalent concentrations of sodium or potassium phosphate a greater inhibition is caused by sodium ion than by potassium ion.[8]

Van Slyke and Cullen[9] observed that the extent of hydrolysis of urea, in an initially unbuffered system, by a crude urease preparation first increased and then decreased upon the addition of increasing concentrations of potassium phosphate and these authors stated that the above effect was due to a change in the *p*H of the reaction system, which apparently was not measured or at least not reported. A reconsideration of the original data has shown that the above explanation

(3) Unpublished data obtained in these laboratories by G. D. Fasman.

(4) H. Lineweaver and D. Burk, THIS JOURNAL, **56**, 658 (1934).

(5) H. E. Armstrong and E. Horton, *Proc. Roy. Soc. (London)*, **85**, 109 (1912).

(6) N. Ondera, *Biochem. J.*, **9**, 544 (1915).

(7) E. F. Armstrong and H. E. Armstrong, *Proc. Roy. Soc. (London)*, **86**, 561 (1913).

(8) See also N. Kato, *Biochem. Z.*, **136**, 498 (1923).

(9) D. D. Van Slyke and G. E. Cullen, *J. Biol. Chem.*, **19**, 211 (1914).

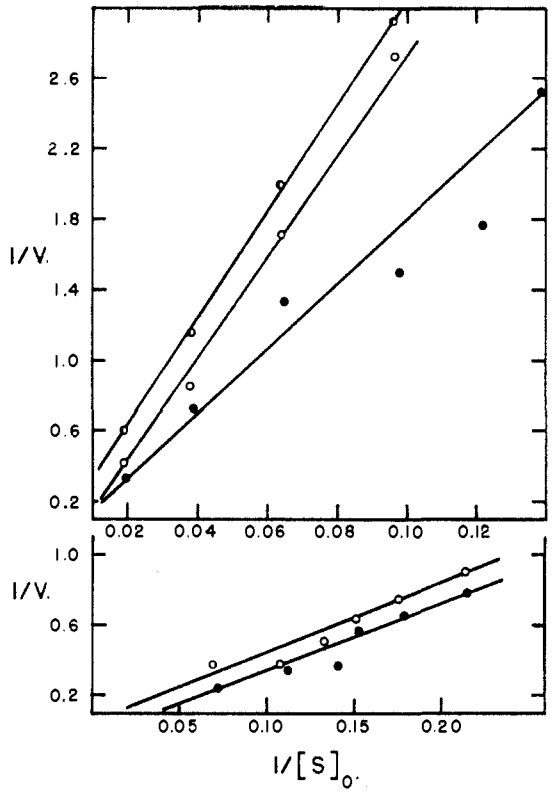

Fig. 3.—Effect of added sodium or potassium chloride upon the activity of urease: $1/v$ in micromoles of ammonia per ml. per min.; $1/[S]_0$ in micromoles urea per ml.; upper plot, all solutions 0.05 molar in sodium phosphate buffer; solid circles, no added sodium chloride; open circles, 0.1 molar in added sodium chloride, half-open circles, 0.265 molar in added sodium chloride; lower plot, all solutions 0.05 molar in potassium phosphate buffer; solid circles, no added potassium chloride; open circles, 0.1 molar in added potassium chloride.

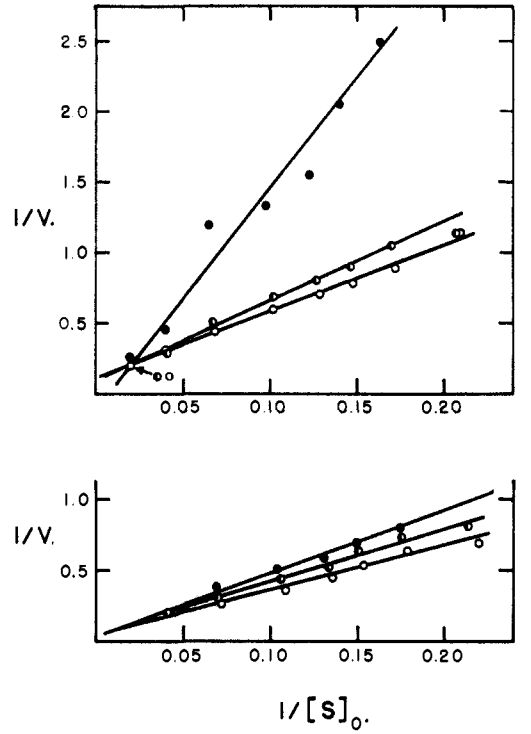

Fig. 4.—Activity of urease with increasing potassium phosphate concentration: $1/v$ in micromoles of ammonia per ml. per min.; $1/[S]_0$ in micromoles of urea per ml.; upper plot, open circles, 0.159 molar phosphate; half-open circles, 0.108 molar phosphate; solid circles, 0.055 molar phosphate; lower plot, open circles, 0.159 molar phosphate; half-open circles, 0.214 molar phosphate; solid circles, 0.267 molar phosphate.

cannot be correct and that the effect observed undoubtedly arose from activation by added phosphate ion and inhibition by added potassium ion. Sizer[10] has noted that urease activity increased and then decreased with increasing concentration of added sodium sulfate and Schmidt[11] found that sodium fluoride caused a greater inhibition of the urease–urea system than did potassium fluoride. Although the former authors' explanation was that the observed effect was due to a change in the E_h of the medium and the latter authors' explanation was based upon an assumed inhibition by fluoride ion it is reasonably clear that here again activation by the added anion and inhibition by the added cation is the more likely explanation.

Within the last year Kistiakowsky and Lumry[12] and Ambrose, Kistiakowsky and Kridl[13] have described experiments which have been interpreted in terms of inhibition of urease by sulfite, bisulfite and phenylsulfinate ions. In view of the fact

(10) I. W. Sizer and A. A. Tytell, *J. Biol. Chem.*, **138**, 631 (1941).
(11) E. G. Schmidt, *ibid.*, **78**, 53 (1928).
(12) G. B. Kistiakowsky and R. Lumry, THIS JOURNAL, **71**, 2006 (1949).
(13) J. F. Ambrose, G. E. Kistiakowsky and A. G. Kridl, *ibid.*, **72**, 317 (1950).

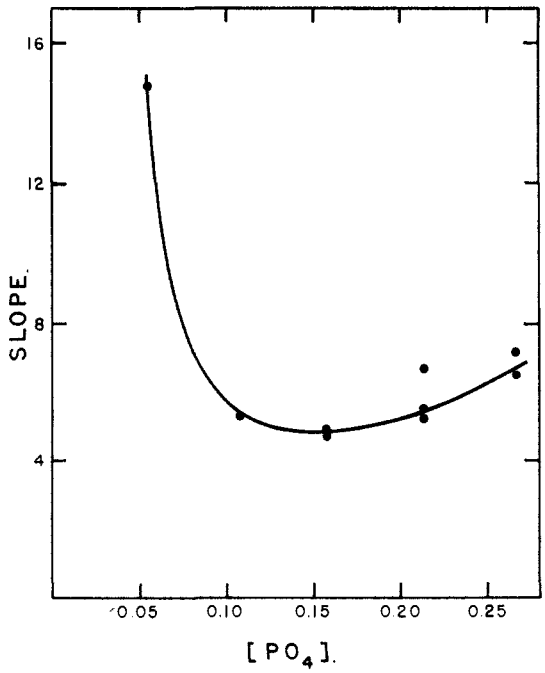

Fig. 5.—Slope of $1/v$ *versus* $1/[S]_0$ plot *versus* potassium phosphate buffer concentration in moles of phosphate per liter.

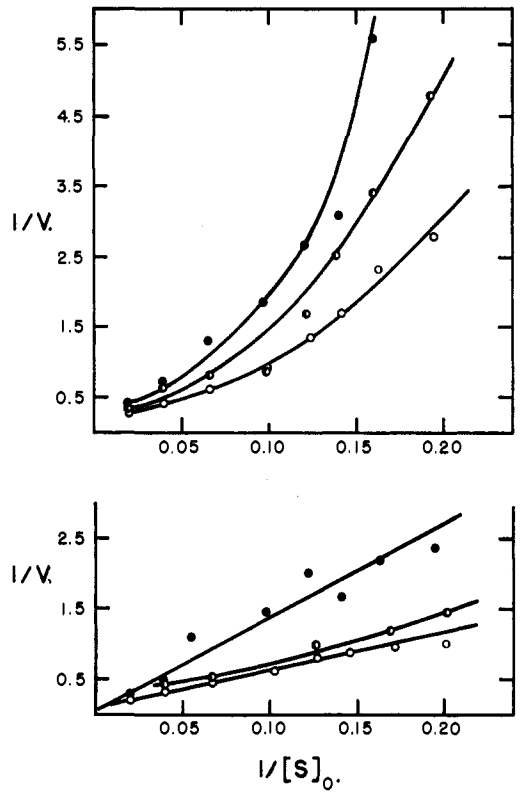

Fig. 6.—Activity of urease with increasing sodium phosphate concentration: $1/v$ in micromoles of ammonia per ml. per min.; $1/[S]_0$ in micromoles of urea per ml.; upper plot, open circles, 0.158 molar phosphate; half-open circles, 0.214 molar phosphate; solid circles, 0.267 molar phosphate; lower plot, open circles, 0.108 molar phosphate, half-open circles, 0.158 molar phosphate; solid circles, 0.055 molar phosphate.

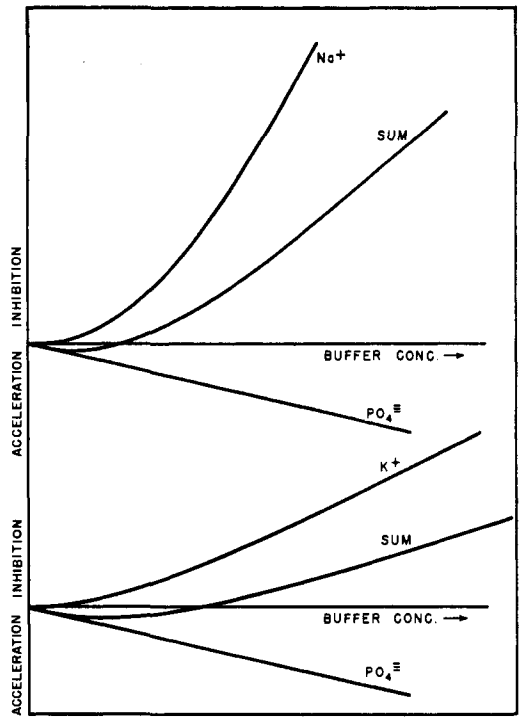

Fig. 7.—Schematic representation of activation and inhibition of urease by sodium and potassium phosphate.

that in the above experiments phosphate ion was also present as was sodium and potassium ion it would appear that the conclusions of these authors require modification. This also appears to be true of the recent studies of Laidler and Hoare[14] on the molecular kinetics of the urease–urea system where the possibility of interaction of the buffer components with the enzyme or enzyme–substrate complex was not considered and where the exact nature of the buffer used was not specified.

In the earlier communication wherein it was erroneously concluded that the urease catalyzed hydrolysis of urea is competitively inhibited by phosphate[2] it was reported that with increasing concentrations of potassium phosphate the slope of the conventional $1/v$ *vs.* $1/[S]_0$ plot increased in a regular manner. The lack of agreement between these observations and those summarized in this communication appears to be due to a difference in the amount of active enzyme present in the respective experiments.[15] The simultaneous activation and inhibition of the urease–urea system by sodium or potassium phosphate may be represented by an arbitrary plot such as that given in Fig. 7. The position of the minimum in the curve representing the net effect of activation and inhibition appears to be dependent upon the concentration of the enzyme, and if the concentration of the enzyme is such as to permit observations to be made only on the ascending branch of the summation curve it is possible, with potassium phosphate buffers where the degree of curvature of this curve appears to be slight, that one could be led to the erroneous conclusion that phosphate is a competitive inhibitor of this enzyme. From information now at hand it is obvious that the term "specific activity," or any of its synonyms, has no general meaning when applied to urease and that a valid analysis of the kinetics of the urease catalyzed hydrolysis of urea not only requires recognition of the phenomena of activation by certain anions and inhibition by certain cations but also knowledge of the concentration of all of the reacting species. Thus it cannot be concluded that the value of 0.16 *M* potassium phosphate, at which maximum activity of urease was observed in the set of experiments reported in this communication (*cf.* Fig. 5), has any independent significance.

In an earlier communication[16] it was noted that the "specific activity" of urease, when determined in systems containing potassium phosphate, increased upon dilution and this observation has since been confirmed in two different laboratories.[13,17] It was originally suggested[16] that the so-called dilution effect was a consequence of either dissociation of urease with an increase

(14) K. J. Laidler and J. P. Hoare, THIS JOURNAL, **71**, 2699 (1949); **72**, 2487, 2489 (1950).

(15) It should be noted that aside from the unknown stoichiometry of the process involving enzyme, activator, substrate and inhibitor an additional uncertainty is introduced by the irreversible inactivation of the enzyme with time.

(16) J. Peterson, K. M. Harmon and C. Niemann, *J. Biol. Chem.*, **176**, 1 (1948).

(17) B. H. J. Hofstee, *J. Gen. Physiol.*, **32**, 339 (1948–1949).

in the number of reactive sites or the dissociation of a urease–inhibitor complex with the inhibitor assumed to be of natural origin. With the recognition that potassium ion can function as an inhibitor and phosphate ion as an activator of urease it appears that neither of the above explanations are correct and that the dilution effect is simply the consequence of a change in the relative concentrations of enzyme, activator and inhibitor.[18,19]

Experimental

Reagents.—The stock 0.1, 0.2, 0.3, 0.4 and 0.5 *M* buffer solutions were prepared from reagent grade dipotassium hydrogen phosphate and potassium dihydrogen phosphate and from the corresponding sodium salts. In every case irrespective of the concentration of the buffer, the *p*H of the solution after final dilution was 7.0 ± 0.02 at 25°. A 1.0 *M* stock solution was prepared daily from urea which had been recrystallized from ethanol. The crystalline urease was prepared from Arlington jackbean meal by the method of Dounce,[20] all operations subsequent to the initial extraction being conducted at 5°. The thrice recrystallized urease obtained from 400 g. of meal was dissolved in 5 ml. of water 1% saturated with hydrogen sulfide at 0° and this stock solution stored at 5°. The water used for the dilution of the enzyme stock solution was also 1% saturated with hydrogen sulfide at 0°. The water used for all solutions was redistilled from an all-glass apparatus.

Procedure.—In general the procedure used was a modification of that described by Van Slyke and Cullen[9] in which the aeration step was eliminated and the ammonia determined by the method of Conway.[21] In practice 2.0-ml. aliquots of one of the above buffer solutions were placed in eight 5.0-ml. volumetric flasks, 1.0 ml. of 0.016, 0.020, 0.028, 0.032, 0.040, 0.060, 0.10 and 0.20 *M* urea solution added to successive flasks and the latter placed in a bath at 25 ± 0.02°. After thermal equilibrium was obtained 0.78 ml. of a diluted enzyme solution was added to each of the above solutions and the mixtures vigorously stirred with a rod kept in each flask. After 3 minutes 0.5 ml. of 2.0 *N* sulfuric acid was added to each flask, the solution again stirred, the flasks withdrawn from the bath, the stirring rods washed and the volume of solution in each flask made up to 5.0 ml. The diluted enzyme solutions were prepared so as to contain approximately 1 microgram of protein nitrogen per ml. of solution. These solutions which were 0.01 *M* in the appropriate buffer were allowed to stand for 5 hours at 25° prior to use. For the determination of liberated ammonia 1.0-ml. aliquots of approximately 0.005 *N* hydrochloric acid containing Tashiro indicator[21] was placed in the central chamber of a Conway dish, a 1.0-ml. aliquot of one of the above 5.0 ml. solutions placed in the outer chamber, the lid, lubricated with glycerol containing sodium hydroxide, placed in position so as to permit the rapid introduction of 1.0 ml. of saturated potassium carbonate into the outer compartment, the dish sealed, the contents in the outer compartment mixed, and the dish allowed to stand overnight at room temperature. The excess acid remaining in the central compartment was then titrated with approximately 0.005 *N* aqueous barium hydroxide. Suitable blanks were provided for each experiment and it was estimated that for a given set of experiments wherein the same enzyme solution was used a precision of ±1.5% was obtained. A least squares treatment was used for the $1/v$ *versus* $1/[S]_0$ plots and in every case $[S]_0$ was taken as the mean substrate concentration prevailing over the 3 minute reaction time. It should be noted that the slopes of the plots given in Figs. 1, 2, 3, 4 and 6 are dependent upon the concentration of active enzyme and because of the irreversible inactivation of urease with time comparisons of the slopes in the above-mentioned figures should be limited to those experiments which were performed simultaneously, *i.e.*, those given in any separate plot. The curve given in Fig. 5, which is based upon data obtained in separate experiments, was constructed by arbitrarily selecting the curve, given in Fig. 4 (upper plot), which has a slope of 4.7 for a phosphate concentration of 0.159 *M* as a standard and adjusting the coördinates of the other plots so that the slope of the curve representing 0.159 *M* phosphate in each of these plots was equal to 4.7.

(18) O. H. Straus and A. Goldstein, *J. Biol. Chem.*, **26**, 559 (1943).
(19) A. Goldstein, *ibid.*, **27**, 529 (1944).
(20) A. L. Dounce, *J. Biol. Chem.*, **140**, 307 (1941).
(21) E. J. Conway, "Micro-diffusion Analysis and Volumetric Error," D. Van Nostrand Co., New York, N. Y., 1940, p. 75.

PASADENA, CALIF. RECEIVED AUGUST 16, 1950

[CONTRIBUTION FROM THE LABORATORIES OF THE SLOAN-KETTERING INSTITUTE FOR CANCER RESEARCH]

A New Synthesis of Purine Nucleosides. The Synthesis of Adenosine, Guanosine and 2,6-Diamino-9-β-D-ribofuranosylpurine[1]

BY JOHN DAVOLL[2] AND BERTRAM A. LOWY

A new synthesis of adenosine, starting from adenine, is presented. Syntheses of 2,6-diamino-9-β-D-ribofuranosylpurine and guanosine from 2,6-diaminopurine are described. The method developed involves the condensation of the chloromercuri derivatives of acylaminopurines with acetylglycosyl halides to give fully acylated aminoglycosylpurines which are converted to the desired nucleosides by deacylation and, in the case of guanosine, deamination. The yields obtainable make this synthetic method suitable for the preparation of isotopically labeled nucleosides.

Previously published work from this Laboratory[3] has dealt with the synthesis of isotopically labeled adenine, hypoxanthine, guanine, isoguanine, xanthine, 2,6-diaminopurine and uric acid for studies of the biosynthesis of nucleic acids. An extension of these metabolism studies to the ribofuranosyl derivatives of these purines would be of obvious interest, and the present communication describes syntheses of adenosine, guanosine and 2,6-diamino-9-β-D-ribofuranosylpurine suitable for the preparation of these compounds isotopically labeled in the purine ring.

Three methods have been reported previously for the synthesis of purine nucleosides.

In the first of these Fischer and Helferich[4] condensed silver 2,8-dichloroadenine with tetraacetylglucosyl bromide and deacetylated the product to give 2,8-dichloro-9-β-D-glucopyranosyladenine. Total reductive dehalogenation of this compound gave 9-β-D-glucopyranosyladenine, while partial reduction gave 2-chloro-9-β-D-glucopyranosyladenine (I, R = β-D-glucopyranosyl). Treat-

(1) The support of the National Cancer Institute of the United States Public Health Service, The Nutrition Foundation, Inc., and the joint support of the Office of Naval Research and the Atomic Energy Commission, contract N6-ori-99, T.O.-1 is gratefully acknowledged.

(2) Public Health Service Postdoctorate Research Fellow of the National Cancer Institute, United States Public Health Service.

(3) For reviews, see G. B. Brown, *Cold Spring Harbor Symposia on Quantitative Biology*, **13**, 43 (1948), and *Federation Proc.*, **9**, 517 (1950).

(4) E. Fischer and B. Helferich, *Ber.*, **47**, 210 (1914).