[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF MICHIGAN, ANN ARBOR, MICHIGAN]

Electrochemical Reduction of Purine, Adenine and Related Compounds: Polarography and Macroscale Electrolysis

BY DAVID L. SMITH AND PHILIP J. ELVING

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The electrochemical reduction of purine and certain derivatives has been examined polarographically, coulometrically and by macroscale reduction over the normal *p*H range. The reduction products have been investigated spectrophotometrically and chemically as well as polarographically. Purine is reduced in two 2*e* stages: the first stage involves reduction of the 1,6 double bond to 1,6-dihydropurine; the second stage involves further reduction, probably to 1,2,3,6-tetrahydropurine, which then hydrolyzes to a 4-aminoimidazole. The product of the first purine 2*e* reduction is slowly oxidized in the presence of oxygen to regenerate purine. Adenine (6-aminopurine) undergoes a single 6*e* reduction, which seems to involve a 2*e* hydrogenation of the 1,6 double bond, followed by the 2*e* reduction of the 2,3 double bond, deamination at the 6-position, further 2*e* reduction of the regenerated 1,6 double bond and hydrolytic cleavage at the 2,3 position to give the same product as in the overall 4*e* purine reduction. Under polarographic conditions the deamination of adenine is negligible, resulting in a 4*e* wave. Hypoxanthine (6-hydroxypurine) apparently only undergoes a 2*e* reduction to 2,3-dihydrohypoxanthine, which then hydrolyzes. Adenine and the completely reduced forms of it and of purine lower the overpotential of hydrogen ion reduction.

Introduction

The polarographic behavior of purine (I of Fig. 1), pyrimidine and their derivatives has been only fragmentarily investigated. The lack of extensive literature in this area, as evidenced by the fact that the electrochemical reduction of purine observed in the present investigation has not been reported previously, is rather surprising in that certain purine and pyrimidine derivatives, being antimetabolites, are of clinical interest in cancer chemotherapy.

Examination of the oxidation–reduction behavior of compounds of biological significance is often of considerable value. Although actual biological redox systems may be of extreme complexity due to enzyme interactions, much information can be obtained from study of the compounds involved in aqueous solution containing inorganic salts with regard to the nature of the factors controlling the electron-transfer processes, *e.g.*, *p*H.

While purine itself has not been investigated polarographically, adenine (6-aminopurine), guanine (2-amino-6-hydroxypurine) and a few other purine derivatives have been studied.[1-5] Unfortunately, two of these studies used only *p*H 1 to 2 solutions; two employed poor polarographic conditions and gave no concentration data; and others did not carefully examine current data. Consequently, it was decided to study systematically purine and several derivatives including adenine, whose study would aid in defining the nature and mechanism of the electrochemical reduction. Controlled-potential reduction at a massive mercury electrode was also employed to determine *n* values (number of electrons transferred per molecule in the electrode process) and their variation with *p*H, and to supply solutions of the reduction products suitable for spectrophotometric and chemical examination.

(1) F. A. McGinn and G. B. Brown, *J. Am. Chem. Soc.*, **82**, 3193 (1960).

(2) J. C. Heath, *Nature*, **158**, 23 (1946).

(3) E. Palacek, *Naturwissenschaften*, **45**, 186 (1958).

(4) N. G. Luthy and B. Lamb, *J. Pharm. and Pharmacol.*, **8**, 410 (1956).

(5) D. Hamer, D. M. Waldron and D. L. Woodhouse, *Arch. Biochem. Biophys.*, **47**, 272 (1953).

Purine

Polarography.—Purine exhibits two waves when in acidic but none when in neutral or alkaline solution. The half-wave potentials of both waves become linearly more negative with increasing *p*H (Fig. 2B); $E_{1/2} = -0.697-0.083\ pH$ for wave I and $-0.902-0.080\ p$H for wave II. The temperature and drop-time dependences of the current indicate diffusion-control. The sum of the diffusion current constants (Fig. 2A) approximately equals that of the single adenine wave under similar conditions. The polarography of purine, especially in respect to the relative magnitude of waves I and II, is subsequently further discussed.

Electrolysis of Wave I.—Macroscale electrolyses at a potential on the crest of wave I give an average coulometric *n* value of 2.2 (Table I). When wave I is eliminated by controlled potential electrolysis, wave II remains unchanged in both current and potential (Fig. 6).

The ultraviolet absorption spectrum of a solution showing only wave II differs considerably from the purine spectrum, *i.e.*, purine (*p*H 5.2): $\lambda_{max} = 263$ mμ, $\log a_M = 3.90$ (literature[6]: $\lambda_{max} = 262.5$, $\log a_M = 3.91$ at *p*H 5.9); after elimination of the first polarographic wave (*p*H 5.2): $\lambda_{max} = 229$ and 292 mμ with $\log a_M = 3.43$ and 3.29, respectively (Fig. 3).

The purine wave I product precipitates with sodium tetraphenylborate (NaTPB), which is a specific precipitant for potassium and quaternary nitrogen bases,[7] whereas an identically treated purine solution (stirred over mercury in the coulometer cell, but not electrolyzed) does not. A polarogram of the filtrate after removal of the wave I product-TPB precipitate and of the excess TPB with KCl, does not show wave II, thus discounting any possibility of wave II being due to nitrogen-free hydrolysis products, *e.g.*, formaldehyde.

Electrolysis of Wave II.—Preliminary coulometry at a potential on the crest of wave II gave an *n* of 9 on complete reduction, which is greatly out of

(6) A. Bendich, P. J. Russell, Jr., and J. J. Fox, *J. Am. Chem. Soc.*, **76**, 6073 (1954).

(7) H. Flaschka and A. J. Bernard, in C. N. Reilley, "Advances in Analytical Chemistry and Instrumentation," Vol. 1, Interscience Publishers, Inc., New York, N. Y., 1960, pp. 1–117.

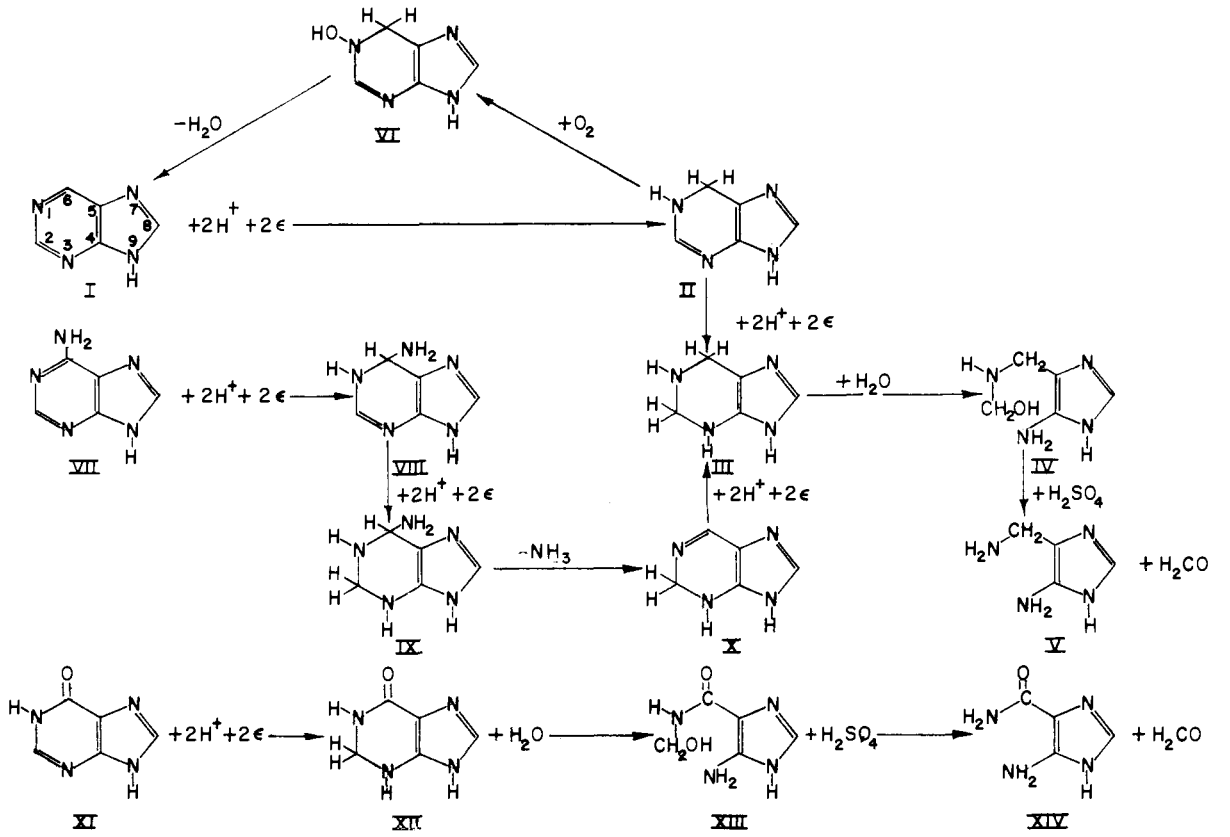

Fig. 1.—Interpretation of the electrochemical and chemical behavior observed for purine (I), adenine (VII) and hypoxanthine (XI), and derived compounds. Protonation, dissociation and other acid–base and keto–enol equilibria are not shown.

TABLE I

COULOMETRIC DETERMINATION OF THE NUMBER OF ELECTRONS INVOLVED IN THE REDUCTION OF PURINE[a]

Concn., mM	Buffer no.	μ, M	pH	Mmoles Electrolyzed	$-E_{max}$, v.	n
			Wave I[b]			
1.25	1	0.50	0.9	0.125	0.80	1.86
1.27	1	.50	1.2	.127	0.80	1.88
1.00	3	.50	4.7	.175	1.13	2.20
1.03	3	.17	4.7	.154	1.12	2.04
1.03	3	.17	4.7	.154	1.12	2.30
1.27	3	.25	4.7	.127	1.11	1.95
			Waves (I + II)[c]			
1.16	3	0.50	4.7	0.116	1.25	4.26, 3.75
1.25	3	.50	4.7	.125	1.25	3.91, 3.63
1.25	3	.50	4.7	.125	1.26	4.22, 3.44
1.25	3	.50	4.7	.125	1.26	4.22, 3.51

[a] A silver coulometer was used. [b] Values of n are corrected for the background electrolyte current as usual. [c] The first value of n in each pair is corrected for the average background electrolyte current; the second represents n corrected for the current blank in the presence of the final concentration of product. The actual n value, consequently, is between these two values since the product concentration is built up during the electrolysis.

proportion to the total I of the waves. Since n, calculated from the ratio of silver deposited to compound electrolyzed (determined from the decrease in wave height), increased with the extent of electrolysis, it appeared likely that hydrogen was being evolved.

Polarographic determination of the decomposition potential confirmed that the purine wave II product, similar to the adenine product discussed later, has a catalytic effect on the evolution of hydrogen. Hydrogen evolution in pH 4.7 acetate buffer, for example, is *ca.* 0.2 v. more positive in the presence of 1.25 mM purine wave II product. The greater the concentration of this product, the more positive is the potential of hydrogen evolution.

Addition of Triton X-100 shifts both purine polarographic waves to more negative potentials by about 20 mv. per 0.002% Triton up to 0.01% Triton. The current of wave I is relatively unaffected, while that of wave II decreases until it approximately equals that of wave I. This behavior would support the hypothesis that adsorption of the wave II product is necessary for the catalytic evolution of hydrogen discussed.

Meaningful coulometric data for wave II (Table I) were obtained when a potential was selected on the rising portion of wave II where the current blank in the presence of purine wave II product (blank A) was as close as possible to that obtained upon electrolysis of the background electrolyte alone (blank B). Four determinations at −1.25 v. in pH 4.7 acetate buffer gave an average n value for the combined purine waves of 3.6 and 4.2, using blanks A and B, respectively. The true blank is actually somewhere between A and B since there is an exponential build-up of the purine wave

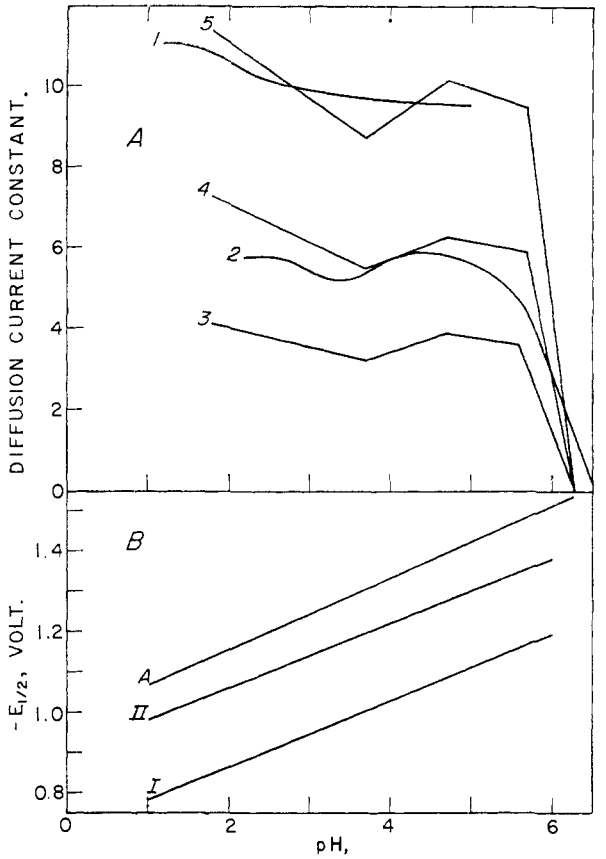

Fig. 2.—(A) Variation of adenine and purine diffusion current constants with *p*H: (1) adenine in chloride and acetate buffers; (2) adenine in McIlvaine buffer; (3) purine wave I in chloride and acetate buffers; (4) purine wave II; (5) total purine diffusion current constant. (B) Variation with *p*H of $E_{1/2}$ of adenine (A), purine wave I and purine wave II.

II product during electrolysis. Consequently, the total reduction of purine can be safely asserted to require four electrons, and each purine wave represents a 2*e* process.

A solution of the wave II product, obtained on elimination of both waves by controlled-potential electrolysis, turns red-purple upon exposure to air when slightly acidic or basic; after a few weeks, the solution becomes yellow-green in color and a finely divided, black, polymeric precipitate forms. Below *p*H *ca.* 2 the product is stable as evidenced by the constancy of the ultraviolet spectrum with time. This behavior is also identical to that observed for the adenine reduction product.

The ultraviolet absorption spectrum of the purine wave II product (Fig. 4) at *p*H 1.5 shows a maximum at 240 mμ (log a_M = 3.54). The wave II product is diazotizable; the diazotized product couples with Bratton–Marshall reagent,[8] N-(1-naphthyl)-ethylenediamine, to yield an orange-red dye (λ_{max} = 503 mμ, log a_M = 4.70) and also with sodium β-naphthoxide to yield an orange-pink dye (λ_{max} = 525 mμ, log a_M = 3.75). The purine wave I product was also tested for a diazotizable amine

(8) A. C. Bratton and E. K. Marshall, Jr., *J. Biol. Chem.*, **128**, 537 (1939).

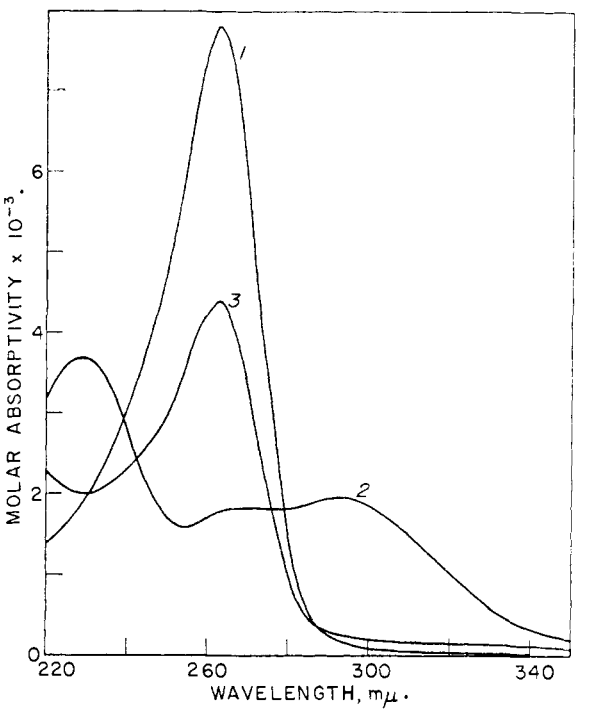

Fig. 3.—Spectra of (1) purine at *p*H 5.2 and of the same solution, (2) immediately after elimination of purine wave I by controlled-potential electrolysis, and (3) after *ca.* 700 hours' exposure to atmospheric oxygen. Concentrations for (2) and (3) are based on that of the purine taken.

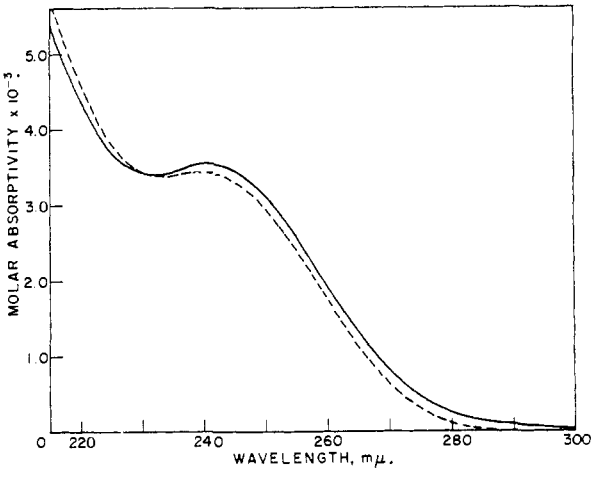

Fig. 4.—Spectra of the purine wave II reduction product (dashed line) and of the adenine reduction product (solid line).

with Bratton–Marshall reagent; the very faint coloration produced undoubtedly resulted from contamination by a small amount of the wave II product, *i.e.*, the potential applied, which was on the crest of wave I, was sufficient to produce a very small amount of the wave II product.

The wave II product gives a positive test for formaldehyde with chromotropic acid, whereas the wave I product and purine do not. Polarography of a solution of the wave II product, buffered to *p*H 11 with LiOH, did not show the

HCHO reduction wave[9]; to insure that the conditions were suitable, a small amount of HCHO was added; the HCHO wave then resulted. The negative polarographic test for HCHO indicates that it is produced from the wave II product under the drastic conditions of the chromotropic acid test,[10] *i.e.*, concentrated sulfuric acid is used. Consequently, the wave II product must be capable of hydrolyzing in concentrated sulfuric acid solution to yield HCHO.

Solutions of the purine wave I and wave II products gave very light yellow precipitates with Nessler's reagent. The nature and color of the precipitates did not compare favorably with that obtained with an equivalent concentration of NH_4Cl. Since many amines give yellow colors and precipitates with Nessler's reagent,[11] the faint yellow precipitate was considered to be a negative test for ammonia.

Regeneration of Wave I.—One of the most interesting aspects of the nature of the product formed in the wave I process and potentially perhaps one of the most significant is its ability to be transformed back to purine. With the passage of time and the exposure of the solution to the atmosphere, wave I is slowly regenerated, while wave II decreases as does also the total current (Figs. 5 and 6); the purine absorption maximum at 263 mμ also returns (Fig. 3).

In a typical experiment, a 1.00 mM purine solution was electrolyzed at a potential on the crest of wave I. The original i_d values were 7.93 and 11.3 μa. for waves I and II, respectively, for a current ratio of 1.43. After electrolysis, wave I was essentially eliminated. The solution, stored in a stoppered flask to prevent evaporation, was then periodically exposed to the atmosphere. After 37 days, the currents of both waves reached equilibrium values (3.5 and 4.9 μa. for a ratio of 1.40), corresponding to a bulk purine concentration of 0.44 mM, *i.e.*, 56% of wave I product had been converted into polarographically nonreducible material, while the remaining 44% had been reconverted to purine.

In order to determine the role of oxygen in the wave regeneration phenomenon, a 1.00 mM purine solution was electrolyzed until wave I was essentially eliminated; the resulting solution was deaerated with argon and the flask flame-sealed. After 20 days the flask was opened and the solution, which was faintly yellow in color, polarographed. There was still only a negligible wave I, but wave II had decreased about 60%. After exposure to air, wave I began to increase, but not as rapidly as in the case where a 1.00 mM reduced solution had been exposed to the atmosphere immediately after electrolysis of wave I.

These results indicate that the rate of regeneration of wave I depends upon the concentration of the wave I product as well as upon the presence of oxygen and that the wave I product slowly decomposes to give polarographically nonreducible material, which imparts a slightly yellow color to the solution. This yellow-colored solution gives a positive test for formaldehyde with chromotropic acid, whereas the test is negative when performed immediately after the electrolysis of wave I.

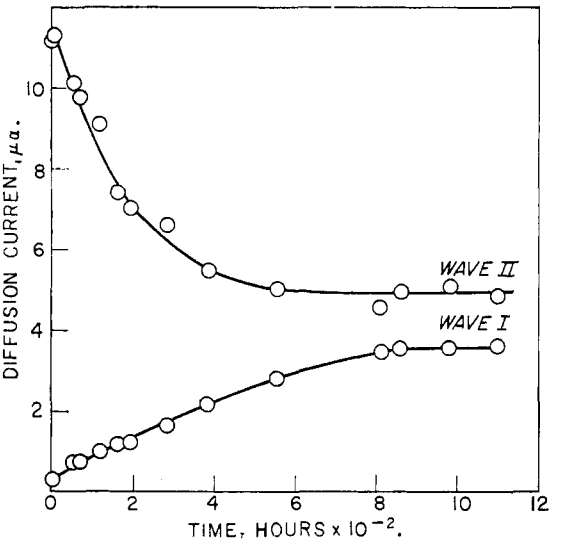

Fig. 5.—Variation upon exposure to air of the heights of the waves of a purine solution following elimination of wave I by controlled-potential electrolysis.

Adenine

Polarography.—Adenine (6-amino-purine, VII of Fig. 1), which was investigated between *p*H 1 and 13, and in unbuffered 0.05 M Bu_4NBr solution, exhibits a wave only in acidic solution. Since the wave occurs close to the background electrolyte decomposition, it is difficult to measure the limiting current accurately, especially since the background discharge occurs at less negative potentials in the presence of adenine.

The drop-time and temperature dependences of the current indicate that the wave is diffusion-controlled. Since the diffusion current constant is *ca.* 40% lower in McIlvaine buffers than in chloride and acetate buffers (Fig. 2A), phosphate and/or citrate may interact with adenine to form loosely bound species having smaller diffusion coefficients than noncomplexed adenine. The presence of adenine lowers the overpotential for hydrogen ion reduction, since even in basic solution, where an adenine reduction wave is not observed, the background discharge occurs at more positive potential than in the absence of adenine. The diffusion current constant in chloride and acetate buffers is 10.2 ± 0.7 at 0.2 mM adenine (Fig. 2A); the approximately linear decrease with increasing adenine concentration (Fig. 7) may be related to the lowering of the hydrogen overpotential by adenine. As the adenine concentration increases, hydrogen is more readily reduced, resulting in a lessening of the demarcation between the adenine and discharge waves.

$E_{1/2}$ varies linearly with *p*H in constant ionic strength McIlvaine buffer[12] (Fig. 2B); $E_{1/2}$ =

(9) I. M. Kolthoff and J. J. Lingane, "Polarography," Vol. II, Interscience Publishers, Inc., New York, N. Y., 1952, p. 652.

(10) P. W. West and B. Sen, *Z. anal. Chem.*, **153**, 177 (1956).

(11) M. J. Taras, in D. F. Boltz, "Colorimetric Determination of Nonmetals," Interscience Publishers, Inc., New York, N. Y., 1958, p. 85.

(12) P. J. Elving, J. M. Markowitz and I. Rosenthal, *Anal. Chem.*, **28**, 1179 (1956).

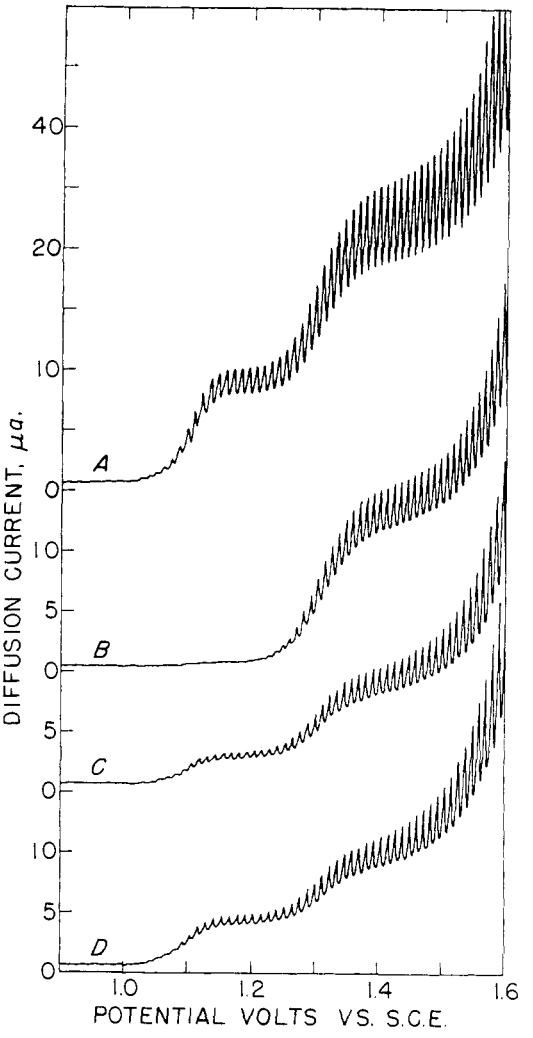

Fig. 6.—Polarograms of (A) 1.00 mM purine in 0.25 M HOAc + NaOAc (pH 4.7), and of the same solution; (B) immediately after electrolysis of wave I; (C) after 385 hours' exposure to atmospheric oxygen; and (D) after 980 hours' exposure.

— 0.975–0.090 pH. $E_{1/2}$ in chloride and acetate buffers agree closely with those in McIlvaine buffer. In pH 1.2 to 2.9 chloride buffer, $E_{1/2}$ becomes more negative with increasing adenine concentration, verifying Heath's observation[2]; $E_{1/2}$ is independent of adenine concentration in acetate buffer.

The possibility that the adenine wave is a catalytic hydrogen wave, rather than being due to reduction of adenine itself, is supported by the large current-concentration ratio, the shift of the wave to more negative potential with increasing adenine concentration and pH, and the presence in adenine of a pyridine-like structure, which is usually associated with catalytic hydrogen wave in acidic solution. However, it is not supported by the mercury-head and temperature dependences of the current. In addition, the wave is independent of the polarization rate, which would not be expected for a catalytic hydrogen wave.[13]

(13) Ref. 9, p. 809.

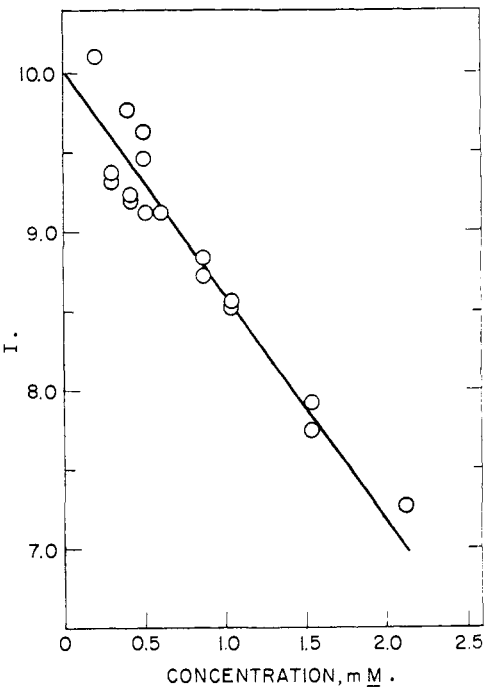

Fig. 7.—Effect of adenine concentration on its diffusion current constant in pH 5.5 acetate buffer.

Coulometry.—Controlled-potential coulometry and large-scale electrolysis definitely show that adenine is reduced at the mercury cathode.

The coulometric runs were followed polarographically; this showed complete elimination of the adenine wave when the electrolysis decreased to background current level, at which point 6 electrons had passed per adenine molecule (Table II). This result is obtained in both chloride and McIlvaine buffers, even though the diffusion current constant in McIlvaine buffers is 40% smaller than in chloride and acetate buffers. The pH change during coulometry in unbuffered solution indicates that at least 5 or 6 hydrogen ions are consumed in the reduction.

TABLE II

COULOMETRIC DETERMINATION OF THE NUMBER OF ELECTRONS INVOLVED IN THE REDUCTION OF ADENINE[a]

Concn., mM[b]	Buffer no.	μ, M	Initial, pH	Final, pH	$-E_{max.}$, v.	n
1.67	2	0.50	2.33	..	1.23	6.25
1.67	2	.50	2.33	..	1.22	5.40
1.67	2	.50	2.33	..	1.23	5.93
1.67	2	.50	2.33	..	1.22	6.30
1.67	1	.17	1.42	1.53	1.12	6.30
1.25	1	.13	1.90	2.21	1.19	5.97
2.50	1	.25	1.60	1.90	1.19	6.28
2.49	1	.25	1.81	4.10	1.33	5.96
1.25	1	.50	1.28	..	1.15	6.38
1.25	1	.50	1.28	..	1.15	6.49
1.25	1	.50	1.28	..	1.15	6.06
					Average	6.12

[a] A silver coulometer was used for the last three runs; the oxygen–hydrogen coulometer was used for the others. [b] The amount of adenine electrolyzed was always 0.125 millimole, except that 0.249 millimole was electrolyzed for the 2.49 mM solution.

Since the product of the adenine reduction exerts a strong catalytic effect on the reduction of hydrogen ion, coulometry of large adenine concentrations is uncertain due to hydrogen evolution. In the coulometric experiments, the potential applied usually corresponded to the $E_{1/2}$ in order to eliminate as much as possible the concomitant evolution of hydrogen, and the results were corrected for background electrolysis (reduction of hydrogen ion) observed in the absence of adenine. With the background electrolytes and the potentials employed for the coulometry of adenine, the concomitant evolution of hydrogen above background current, for which correction was made, is negligible, *i.e.*, the background current blanks determined both in the presence and in the absence of product were essentially identical.

The reduced adenine solution turns red-purple upon exposure to air when slightly acidic or basic; a finely divided black precipitate forms upon long standing. Reduction of adenine with zinc and HCl gives a solution of similar appearance, which also precipitates on standing.[14] Further evidence for reduction is supplied by the ultraviolet spectra before and after reduction, *i.e.*, adenine (*p*H 5.2): λ_{max} = 262.5 mμ, log a_M = 4.111 (literature,[15] λ_{max} = 264 mμ, log a_M = 4.098 at *p*H 2); after complete reduction of adenine: λ_{max} = 240 mμ and log a_M = 3.55 at *p*H 1.4 (Fig. 4).

When a reduced adenine solution is made basic with NaOH and about one-half the contents distilled into dilute HCl solution, evaporation of the latter to dryness, leaves a white solid which completely sublimes upon heating. Collection and analysis of this sublimate shows it to be NH_4Cl. Further evidence for the formation of ammonia is given by a positive test with Nessler's reagent on the reduced solution.

More positive evidence about the nature of the reduction products is given by examination of the precipitates obtained with sodium tetraphenylborate with a reduced adenine solution; an identically treated adenine solution (stirred over mercury in the coulometer cell, but not electrolyzed), does not give a precipitate. The TPB precipitate, originally white in color, turns purple upon standing in air; the rate of this color transformation is increased by heating; consequently, the TPB precipitate was dried in a vacuum desiccator over P_2O_5.

The weight of TPB precipitate obtained in a series of four electrolyses from electrolysis of 0.250 millimole of adenine in *p*H 5 acetate buffer was 187 ± 7 mg., indicating that at least two quaternary nitrogens are involved in the precipitate. Comparison of this weight of precipitate with that expected for various types and combinations of products which might be produced from 0.250 millimole of adenine, indicates that the best concordance is that for an equimolar mixture of ammonia and amine, where the latter is in the molecular weight range of an imidazole derivative formed by fission of the pyrimidine ring of the adenine. Air oxidation of this derivative then accounts for the TPB precipitate turning purple on exposure to air.

Undoubtedly, hydrolysis-type reactions follow the electrochemical reaction, *e.g.*, the reduced adenine solution gives a positive test for a diazotizable amine. The adenine reduction product couples with Bratton–Marshall reagent after diazotization to yield an orange-red dye (λ_{max} = 503 mμ, log a_M = 4.64) and with sodium β-naphthoxide to yield an orange-pink dye (λ_{max} = 525 mμ, log a_M = 3.80). Adenine reduced with zinc and sulfuric acid also gives an orange-red dye (λ_{max} = 503 mμ) after diazotization and coupling with Bratton–Marshall reagent.[16]

The adenine reduction product gives a positive chromotropic acid test for formaldehyde, while adenine itself does not. Since a polarographic wave was not observed for formaldehyde under conditions suitable for formaldehyde reduction (*vide supra*), the adenine product must be capable of hydrolyzing in concentrated sulfuric acid to yield formaldehyde.

Hypoxanthine.—Polarograms of 0.5 m*M* hypoxanthine (6-hydroxypurine, XI of Fig. 1) at *p*H 2.0 (buffer 1), 3.7 (buffer 3) and 4.7 (buffer 3) showed very ill-defined inflections on the background discharge, whose potential was *ca.* 0.1 v. more positive than in the absence of hypoxanthine. At *p*H 5.7 (buffer 3) a fairly well-defined limiting current was obtained after point-by-point subtraction of the residual current (I = 2.75; $E_{1/2}$ = − 1.61). An inflection was not observed in neutral solution or *p*H 9.2 ammonia buffer.

Guanine.—Guanine (2-amino-6-hydroxypurine) is very insoluble in acidic and neutral solution, especially in the presence of relatively large concentrations of buffer components; consequently, it was necessary to polarograph saturated solutions. However, it did not give any indication of a polarographic wave in the Table III buffers or 0.05 *M* Me_4NBr solution. Since guanine is resistant to chemical reduction with zinc and HCl,[5] it is not surprising that it cannot be reduced under normal polarographic conditions.

Mechanism of Purine Reduction

The electrochemical reduction of purine and adenine clearly involves hydrogenation and finally fission of the pyrimidine portion of the ring; the latter is supported by the facts that imidazole has a benzene-like inertness[17,18] and is not reduced polarographically[19] and that pyrimidine is polarographically reduced.[19] Before considering the evidence for the mechanisms of electrochemical reduction of these and related compounds, which mechanisms together with related chemical reactions are summarized in Fig. 1, it is helpful to evaluate the effects of substituents on the reduction.

(14) A. Kossel, *Z. Physiol. Chem.*, **12**, 241 (1888).

(15) L. F. Cavalieri, A. Bendich, J. F. Tinker and G. B. Brown, *J. Am. Chem. Soc.*, **70**, 3875 (1948).

(16) S. Friedman and J. S. Gots, *Arch. Biochem. Biophys.*, **39**, 254 (1952).

(17) K. Hofmann, "Imidazole and Derivatives," Part I, in A. Weissberger, "The Chemistry of Heterocyclic Compounds," Interscience Publishers, Inc., New York, N. Y., 1953.

(18) E. S. Schipper and A. R. Day, "Imidazoles and Condensed Imidazoles," in R. C. Elderfield, "Heterocyclic Compounds," Vol. V, John Wiley and Sons, New York, N. Y., 1957, Chapter 4.

(19) L. F. Cavalieri and B. A. Lowy, *Arch. Biochem. Biophys.*, **35**, 83 (1952).

Effect of Substituents.—An apparent correlation has been noted[4] between the presence of an amino group in the 6-position of the purine ring and the appearance of a polarographic wave. However, since purine itself is polarographically reducible, the 6-amino group is obviously not a necessary criterion for reduction of a purine; in fact, the presence of the 6-amino group makes reduction more difficult. In general, the ease of reduction decreases with the number of substituents added to the pyrimidine portion of the purine. The effect of amino and hydroxy substituents seems to involve saturation of the ring by means of tautomeric shifts, thereby removing reduction sites, *i.e.*, double bonds in the ring.

TABLE III

BUFFER AND BACKGROUND ELECTROLYTE SOLUTIONS[a]

Buffer no.	*p*H	Composition
1	0.4– 2.9	KCl or NaCl + HCl
2	2.2– 8.0	$Na_2HPO_4 \cdot 7H_2O$. citric acid monohydrate + KCl
3	3.7– 5.7	NaOAc + HOAc
4	6.1– 7.0	KCl
5	8.5– 9.2	$NH_4Cl + NH_3$
6	11.0–13.2	KCl + NaOH

[a] All buffer solutions were used at an ionic strength of 0.5 *M* unless otherwise indicated.

A 6-amino group promotes reduction only in the sense that the resulting compound is easier to reduce than one substituted with a hydroxy group in this position, *e.g.*, compare adenine and hypoxanthine.

Since adenine and 2,6-diaminopurine have similar half-wave potentials,[4] the 2-amino group seems to be relatively ineffective as a potential-controlling substituent; the additional fact that the two compounds produce very similar currents also tends to indicate that the reduction process is the same.

Acetyladenine (6-(N-acetylamino)-purine) gives two reduction waves.[4] It is doubtful that the first wave is due to the reduction of the acetyl group, but rather the presence of this group on the amino group promotes a 2*e* reduction of the purine ring at the 1,6 position. This is indicated by the fact that adenine and acetyladenine give approximately equal total currents, whereas the current for 2,6-bis-(N-acetylamino)-purine is apparently the same as that of acetyladenine. Furthermore, N^2-acetylguanine does not give a wave,[4] as might be expected if the acetyl group itself were directly involved. Presence of this group in acetyladenine seems to counterbalance somewhat the effect of the amino group, and the behavior of acetyladenine is similar to that of purine.

Purine.—Wave I of purine is a 2*e* process and suggests hydrogenation of a double bond. The presence of a diazotizable, *i.e.*, primary aromatic, amine in the products from completely reduced adenine and purine shows that the 4,5 double bond, which is in the imidazole ring, is unaffected. Only two other reduction sites remain: the 2,3 and 1,6 double bonds.

A primary aromatic amino group can only result from the N-3 nitrogen, since the N-1 nitrogen is not directly attached to the imidazole ring; formation of this amine therefore requires fission of the 2,3 double bond. Since purine can be regenerated by oxygen from the purine wave I product, it is improbable that ring fission occurs during the wave I process. Moreover, the fact that the diazotizable amine is not produced during the wave I process also suggests that the 1,6 double bond reduction is the energetically more favorable one.

Association of the first purine reduction step with the 1,6 double bond to form 1,6-dihydropurine (II of Fig. 1) is also supported by the following: (a) addition of a 6-amino group to purine to give adenine profoundly affects the ease of reduction, while further addition of a 2-amino group has little effect, (b) an acetyl group on the 2-amino group of 2,6-diaminopurine is ineffective, while one on the 6-amino group alters the polarographic behavior,[4] (c) N,N′-diphenylacetamidine, which contains the 2,3,4,5 system of purine is not reduced polarographically[20] and (d) purine is catalytically hydrogenated to 1,6-dihydropurine.[21]

Wave II would then seem to result from a further 2*e* reduction to 1,2,3,6-tetrahydropurine (III), which on subsequent hydrolysis would form [(5-aminoimidazol-4-ylmethyl)-amino]methanol (IV), which would account for the diaotizable amine observed. 4(or 5)-Amino-substituted imidazoles, unlike most other imidazoles, are highly unstable and have only been isolated as salts, *e.g.*, short exposure of 4(or 5)-aminoimidazole to room temperature leads to its decomposition with the formation of black pigments[22]; similar behavior is observed for compound IV in slightly acidic or basic solution.

Subsequent discussion regarding the mechanisms of adenine and hypoxanthine reduction will provide further evidence for the presence of IV and V. Compound IV would be expected to hydrolyze in concentrated sulfuric acid to yield 4(or 5)-amino-5-(or 4)-(aminomethyl)-imidazole (V) plus formaldehyde, thus accounting for the positive chromotropic acid test. The higher stability of IV, observed near *p*H 2, undoubtedly results from protonation of the 4(or 5)-amino group.

A likely mechanism for the regeneration of purine by oxygen from 1,6-dihydropurine involves the formation of 1-hydroxy-2-hydropurine (purin-1-(6H)-ol) (VI), which subsequently dehydrates to regenerate the stable purine ring.

Adenine.—The ultraviolet absorption spectrum of the adenine reduction product at *p*H 1.4 is essentially superimposable on that of the purine wave II reduction product at *p*H 1.5 (Fig. 4), indicating identity of the two products.[23] Identical spectra are also obtained for the dyes formed after diazotization of the two reduction products with Bratton–Marshall reagent and sodium β-naphthoxide (the small differences in the a_M values may be due to incomplete diazotization).

(20) M. E. Runner, M. L. Kilpatrick and E. C. Wagner, *J. Am. Chem. Soc.*, **69**, 1406 (1947).

(21) A. Bendich in "Chemistry and Biology of Purines," Little, Brown and Co., Boston, Mass., 1957, pp. 308–310.

(22) Ref. 17, pp. 141–143.

(23) The small difference in the two spectra is probably due to the small difference in the base line.

Formation of identical products in the adenine and purine wave II processes requires elimination of the 6-amino group from adenine; this would account for the formation of ammonia during the adenine reduction.

The first (potential-controlling) step in the adenine reduction probably involves hydrogenation of the 1,6 double bond. This is supported not only by the purine results and the profound effects produced by substituents added to the 6-position, but also by the fact that 5-amino-4-imidazolecarboxamidine, which does not contain the 2,3 double bond, exhibits an $E_{1/2}$ of -1.57 v. at *p*H 6.8,[19] which is in conformity with the adenine $E_{1/2}$–*p*H curve (Fig. 2B).

The sequence of the steps following the 1,6 double bond reduction cannot be ascertained with certainty, but the formation of ammonia, the 6*e* nature of the wave and the identity of the purine wave II and adenine reduction products require (a) reduction of the 2,3 double bond, (b) deamination at the 6-position, (c) further reduction of the regenerated 1,6 double bond and (d) hydrolytic cleavage at the 2,3 position. The chemical steps, deamination and hydrolysis, are undoubtedly slower than the electrochemical ones. Hydrogenation of the 2,3 double bond as the second step under polarographic conditions would result in 1,2,3,6-tetrahydroadenine (IX) and would account for four electrons. Deamination of the latter would result in 2,3-dihydropurine (X). Reduction of the regenerated N=C—C=C— system at the 1,6 double bond would result in (III), which subsequently hydrolyzes to yield (IV). This process accounts for the over-all 6-electron nature of the process as determined by controlled-potential electrolysis and for the formation of the same diazotizable amine as produced in the purine reduction.

It is difficult to say whether under the conditions of controlled-potential electrolysis reduction and subsequent hydrolysis of the 2,3 bond precedes or follows deamination of the 6-amino group; very probably, these reactions proceed simultaneously. Deamination may be aided by the electrostatic attraction of the protonated amino group to the electrode. Under polarographic conditions, however, it is doubtful that deamination proceeds appreciably, since adenine and purine give approximately identical total currents.

The appearance of polarographic maxima on the purine wave II and the adenine wave at low *p*H may be due to the increased rate of hydrolysis at the reduced 2,3 bond. A chemical reaction proceeding at the electrode surface may result in microscopic concentration gradients with concomitant stirring.

Hydroxypurines.—Reduction of hypoxanthine (6-hydroxypurine) very likely involves the 2*e* hydrogenation of the 2,3 double bond to form 2,3-dihydrohypoxanthine (XII), which would then hydrolyze to 4(or 5)-amino-5(or 4)-N-(hydroxymethyl)-imidazolecarboxamide (XIII); the 1,6 double bond is unavailable for reduction due to the stability of the keto form. Further hydrolysis in strongly acidic solution would result in formaldehyde and 5-amino-4-imidazolecarboxamide (XIV). The latter has been identified[16] as a product in the zinc reduction of hypoxanthine; the other closely related amine observed may have been XIII.

2-Hydroxypurine, which has not been investigated polarographically, can be predicted to have an $E_{1/2}$ close to that of purine on the basis that substituents in the 2-position have a minor effect upon the ease of reduction,[24] and both purine and 2-hydroxypurine absorb one mole of H_2 per mole of compound, whereas adenine and hypoxanthine are unaffected.[6]

Experimental

Chemicals.—Elemental and spectrophotometric analysis and chromatographic assay showed the adenine and guanine (Nutritional Biochemicals Corp.) and purine and hypoxanthine (Mann Research Laboratories) to be of sufficient purity for polarographic study. Aqueous stock solutions of the polarographically-active purines were stable, as evidenced by the constancy of their reduction currents with time.

Buffer solutions (Table III) were prepared from analytical reagent grade chemicals. The nitrogen used for deoxygenating was purified and equilibrated by bubbling it successively through alkaline pyrogallol solution, sulfuric acid and distilled water.

Apparatus.—Polarograms were recorded on a Leeds & Northrup Type E Electro-chemograph, using a water-jacketed H-cell,[25] maintained at 25.0 ± 0.1°, unless otherwise specified, and containing a saturated calomel reference electrode (s.c.e.). Damping of the polarograph was equivalent to galvanometer performance.

Capillaries (marine barometer tubing) had the following *m* values in mg./sec. in distilled water (open circuit) at 25°: (A) 2.69 at 30 cm. of mercury; (B) 1.85 (40 cm.); (C) 3.07 (60 c.m); (D) 2.18 (40 cm.) and 3.23 (60 cm.). Drop-times, measured at the potentials of interest, were generally between 3 and 4 seconds.

The cell resistance, measured with a General Radio Type 650A impedance bridge, was always below 300 ohms (usually below 150); consequently, half-wave potentials were not corrected for *iR* drop.

The coulometric cell[26] had a stirred massive mercury cathode, a large external working silver anode (a coil of about 100 cm. of No. 10 gage silver wire immersed in saturated KCl solution) connected to the cell *via* a KCl salt bridge and a s.c.e. reference electrode. In all experiments where tetraphenylborate was used, the KCl in the reference electrode and silver anode compartment was replaced with NaCl and the background electrolyte was either NaCl + HCl or NaOAc + HOAc. The cell was connected in series with either an oxygen–hydrogen or a silver coulometer, and the Fisher Controlled Potential Electro-Analyzer, which was used to control automatically the potential. The potential between mercury cathode and reference electrode was checked with a Rubicon Type B-1 potentiometer.

Ultraviolet spectra were obtained with Cary Model 11 and Beckman DU spectrophotometers, using 1-cm. stoppered quartz cells. *p*H was measured with a Leeds & Northrup No. 7664 *p*H meter.

Polarographic Procedure.—The test solution was usually prepared by diluting a known volume of stock solution to 50.0 ml. in a volumetric flask with the desired buffer; the *p*H of this solution was measured. About 10 ml. of test solution was transferred to the H-cell, deoxygenated with nitrogen for about 10 minutes and then polarographed. Triton X-100 (concentration of 0.002%) was used in a few cases to suppress maxima. A portion of the buffer solution was treated in identical fashion to obtain the background curve. The residual current was subtracted arithmetically from the total current; $E_{1/2}$ and i_d were determined graphically, utilizing the average of the recorder trace.

(24) D. L. Smith and P. J. Elving, unpublished work.

(25) J. C. Komyathy, F. Malloy and P. J. Elving, *Anal. Chem.*, **24**, 431 (1952).

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Coulometric Procedure.—Fifty ml. of buffer solution was added to the coulometer cell and deoxygenated for about 10 minutes; the mercury was then introduced and the solution electrolyzed at a potential more negative than at which the electrolysis was to be run, until the current fell to its minimum value, generally 1 to 2 ma. The mercury was removed; a known volume of a stock solution containing the electroactive species added; nitrogen was again bubbled through the solution; the mercury reintroduced; the coulometer connected (the hydrogen–oxygen coulometer was saturated with the gases prior to use); and electrolysis at the desired controlled potential started. Nitrogen was passed continuously through the cell during electrolysis.

Solutions appropriate for spectrophotometry were prepared by dilution with water to an appropriate volume followed by *p*H measurement. The identical concentration of buffer and *p*H were employed in the reference cell.

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[CONTRIBUTION FROM THE BIOCHEMISTRY DEPARTMENT, UNIVERSITY OF PITTSBURGH SCHOOL OF MEDICINE, PITTSBURGH, PENNSYLVANIA]

Insulin Peptides. III. Synthesis of a Protected Nonapeptide Containing the C-Terminal Sequence of the B-Chain of Insulin[1]

BY PANAYOTIS G. KATSOYANNIS AND KENJI SUZUKI[2]

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The protected heptapeptide N-carbobenzoxy-L-phenylalanyl-L-phenylalanyl-L-tyrosyl-L-threonyl-L-prolyl-N$^{\epsilon}$-tosyl-L-lysyl-L-alanine methyl ester was prepared by stepwise elongation of the peptide chain from the amino end. Decarbobenzoxylation of this compound and coupling of the resulting product with N$^{\alpha}$-carbobenzoxy-N$^{\omega}$-nitro-L-arginylglycine *p*-nitrophenyl ester gave the nonapeptide N$^{\alpha}$-carbobenzoxy-N$^{\omega}$-nitro-L-arginylglycyl-L-phenylalanyl-L-phenylalanyl-L-tyrosyl-L-threonyl-L-prolyl-N$^{\epsilon}$-tosyl-L-lysyl-L-alanine methyl ester. This protected nonapeptide contains the C-terminal amino acid sequence of the B-chain of insulin.

Degradative studies by Sanger and co-workers[3] led to elucidation of the amino acid sequence of insulin and the postulation of a complete structure for this hormone. In this structure the amino acid sequence. . .-arginyl-glycylphenylalanyl-phenylalanyl-tyrosyl-threonyl-prolyl-lysyl-alanine occupies the C-terminal position of the B-chain of the insulin molecule.

In continuation of our studies of synthetic polypeptides with amino acid sequences found in insulin,[4,5] we undertook the synthesis of a protected nonapeptide (VII) embodying the above mentioned amino acid sequence of the C-terminal region of the insulin B-chain.

The synthesis of this peptide was accomplished by a combination of two principal synthetic approaches, *viz.*, "stepwise elongation" and "fragment condensation."[4] The protected C-terminal heptapeptide,[6] N-carbobenzoxy-L-phenylalanyl-L-phenylalanyl-L-tyrosyl-L-threonyl-L-prolyl-N$^{\epsilon}$-tosyl-L-lysyl-L-alanine methyl ester (VI), was prepared by stepwise elongation of the peptide chain from the amino end. The appropriate N-carbobenzoxy amino acids which served as the "carboxyl component" in each step were activated either by the use of N,N′-dicyclohexylcarbodiimide[7] or by conversion to the corresponding *p*-nitrophenyl esters.[8] In the latter case, aminolysis of the *p*-nitrophenyl esters was carried out in dimethylformamide for 24 hr. The reaction mixture was then treated with 1 *N* NH_4OH to destroy the excess *p*-nitrophenyl ester and the product isolated in the manner described in the experimental section. The yields of the various synthetic steps ranged from 71 to 96% of the theoretical. Leucine amino peptidase (LAP) digestion of the decarbobenzoxylated heptapeptide ester followed by paper chromatography revealed the presence of ninhydrin positive components with R_f values corresponding to the expected amino acids. Ninhydrin reactive components with R_f values corresponding to any of the intermediates were absent. This suggests that the digestion was complete and indicates[9] that the optical homogeneity of the constituent amino acids was preserved during the synthesis of the heptapeptide fragment. The protected dipeptide, N$^{\alpha}$-carbobenzoxy-N$^{\omega}$-nitro-L-arginylglycine,[10] which contains the amino acid sequence found in the N-terminal region of the nonapeptide, was converted to its *p*-nitrophenyl ester and subsequently coupled with the decarbobenzoxylated heptapeptide to give the desired protected nonapeptide N$^{\alpha}$-carbobenzoxy-N$^{\omega}$-nitro-L-arginylglycyl-L-phenylalanyl-L-phenylalanyl-L-

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