19 October 1956, Volume 124, Number 3225 SCIENCE

Enzymatic Oxidation of Urate

H. R. Mahler, Harold M. Baum, Georg Hübscher

Although the enzyme uricase (uricooxidase) has now been known for some 50 years (*1*) and numerous extensive purification procedures have been described in the literature (*2, 3*), its mode of action and even the exact nature of the reaction it catalyzes has remained somewhat obscure until fairly recently. Investigations concerning the mechanism may be said to have had their inception with the demonstration by Schuler (*4*) that oxidation of urate (U^-) may occur without the simultaneous liberation of carbon dioxide. Praetorius (*5*) provided evidence that in nonborate buffers at least two short-lived but spectroscopically distinct complexes intervened between urate and allantoin, while a different path was followed in borate buffer. Bentley and Neuberger (*6*) showed conclusively by means of isotopic tracers that all the carbon dioxide had its origin in carbon atom No. 6 (C-6) of the uric acid molecule and that the enzyme functioned as an aerobic dehydrogenase, inasmuch as none of the oxygen used in the reaction was incorporated into the product allantoin. Thus, the reaction catalyzed by the enzyme was formulated as follows (*6*):

$$\begin{array}{l} \overset{-2e^-}{U^- \rightarrow U^+} \rightarrow I \rightarrow II \rightarrow \text{allantoin} + CO_2 \\ \qquad\quad \downarrow \text{borate} \\ \qquad\quad X \\ O_2 + 2e^- + 2H^+ \rightarrow H_2O_2 \end{array} \qquad (1)$$

The enzyme has now been obtained in good yield from pig-liver particles and purified to a state approaching homogeneity (*7*). Thus it has been possible to investigate several of the outstanding problems concerning its mode of action (*8*). This report is a summary and extension of some of these findings (*9*).

Active Site on the Enzyme

Uricase was identified as a cuproprotein containing 0.05 percent copper, corresponding to 1 gram atom of the metal per mole of enzyme (~ 120,000 grams). The metal is bonded very tenaciously by the enzyme protein and cannot be dissociated even in 5 percent trichloroacetic acid or by dialysis against strong copper-chelating agents (*10*). Thus it has not been possible to conduct experiments on the reversible dissociation of the holoenzyme and reactivation of the apoenzyme. Nevertheless, copper has been assigned as one of the sites binding the substrate to the enzyme on the basis of the following evidence. (i) There is parallelism of increase in copper content and specific enzymatic activity during purification (*7*). (ii) Urate, like other purines, is capable of chelation with metals (*11*); among these chelates, those of copper are among the most tightly bound (*12, 13*). (iii) The enzyme is inhibited by cyanide (*2*) and by other metal-complexing agents, which, like cyanide, are also capable of acting as reducing agents (*7*). (iv) These inhibitions are either completely or partially overcome in the presence of urate at low concentration (*7*). (v) The spectroscopically identifiable complex of diethyl,dithiocarbamate, copper, and enzyme, which is formed by dialysis of the enzyme against the chelating agent, is dissociated in the presence of urate. The linkage to copper is believed to be through chelate bonds to oxygen at C-6 and nitrogen atom No. 7 (N-7) by analogy with similar heterocyclic systems (*11*).

The kinetics of the enzyme-catalyzed reaction have been studied extensively, and some of the characteristic constants are summarized in Table 1. Additional linkage sites on the enzyme have been suggested from an examination of the *p*H dependence of the Michaelis constant and the maximal velocity (*14*) of the enzyme-catalyzed disappearance of urate. The two sites, one acidic and one basic, have pK_a's of 7.5 and 9.2, respectively, and have been tentatively identified as an α-amino and an ε-amino (or phenolic hydroxy) group of the protein (*12, 15*). Confirmation of this postulate and additional information have been derived from a study of the inhibition of enzymatic action exerted by a large number of 2,6,8-trisubstituted purines. This inhibition was found to be competitive to urate in all cases where it occurred at all (see Table 2) and thus permitted us to draw conclusions with respect to the structural parameters necessary for the formation of an effective enzyme-inhibitor complex.

Thus two binding sites, one cationic and linking the protein to the substituent at C-2, and one neutral, providing the link between protein and the substituent at C-8, have been identified. It is tempting to equate these two binding sites with the ε-amino (or phenolic hydroxyl) and α-amino group that we referred to in the previous paragraph.

Products and Intermediates

The availability of the highly purified enzyme has permitted us to establish the stoichiometry of the over-all reaction carried out by the enzyme. In phosphate or tris(hydroxymethyl)aminomethane buffers in the *p*H range between 7.0 and

Dr. Mahler, former assistant professor of enzyme research at the Institute for Enzyme Research, University of Wisconsin, is now on the staff of the department of chemistry at Indiana University. Dr. Baum and Dr. Hübscher were postdoctoral trainees of the National Heart Institute, National Institutes of Health, U.S. Public Health Service, at the Institute for Enzyme Research. Dr. Baum is now on the staff of the department of biochemistry, University of Cardiff, Wales. Dr. Hübscher is now on the staff of the department of pharmacology, University of Birmingham, England.

9.0, this stoichiometry is strictly that of reaction 2:

$$U^- + O_2 + 3H_2O \xrightarrow{\text{uricase}} \text{allantoin} \cdot H_2O_2 + HCO_3^- \quad (2)$$

The product is written as a stoichiometric complex of allantoin and hydrogen peroxide, inasmuch as mild treatment permits its isolation and recrystallization. Upon chromatography, solvent extraction or, most efficiently, treatment with catalase, the complex is decomposed with the resulting production of allantoin. In practice, for the large-scale isolation and identification of allantoin, the oxidation is performed in the presence of catalase and ethanol (*16*). Under these conditions the reaction measured becomes reaction 3:

$$U^- + \tfrac{1}{2}O_2 + \text{ethanol} + 2H_2O \xrightarrow[\text{catalase}]{\text{uricase}} \text{allantoin} + HCO_3^- + \text{acetaldehyde} \quad (3)$$

We have been able to confirm the complete conversion of urate C*-6 to $HC^*O_3^-$ (*6*) and the equally complete retention of C*-2 and C*-8 in allantoin (*17*).

In borate buffer, in addition to reaction 2, urate is oxidatively decomposed by alternative reactions. Cannelakis and Cohen (*19*) have shown that the other main reaction is as follows:

$$U^- + O_2 + 3H_2O \xrightarrow{\text{uricase}} UIDC^- + H_2O_2 \quad (4)$$

The product UIDC (5-ureido-2-imidazolidone-4,5-diol-4-carboxylate) is unstable, especially in the absence of borate, and decomposes according to reaction 5:

$$UIDC \longrightarrow \text{alloxanic acid} + \text{urea} \quad (5)$$

By the use of relatively rapid spectrophotometric techniques in studies of the possible intermediates during uric acid oxidation, we have made the following determinations. (i) The formation of intermediates I and II (*5*) is confirmed. (ii) At low urate concentrations, intermediate I is formed enzymatically by first-order kinetics; its rate of appearance equals exactly the rate of urate disappearance. (iii) When enzyme action is stopped—for example, in the presence of 10^{-3} *M* cyanide—the formation of intermediate I ceases immediately, and its rate of disappearance may then be studied without additional complications. (iv) Intermediate I is decomposed spontaneously and reversibly to intermediate II in a nonenzymatic reaction; this reaction is first order with respect to intermediate I and is *p*H-dependent. (v) Intermediate I is formed in borate buffer as well as in nonborate buffers, but its first-order rate of disappearance in the former is vastly enhanced, especially at moderate *p*H; II is not an intermediate in the borate-sensitive reaction. (vi) During the initial phases of the reaction in nonborate buffers, all the urate added can be accounted for as unchanged urate, as intermediate I, or as intermediate II (assuming approximately equal ε_{max} for all three compounds).

The ultraviolet absorption spectra of intermediates I and II have been studied in some detail. Intermediate I (λ_{max} = 305 mμ; $\varepsilon_{max} = \simeq 10{,}000$) shows two "spectrophotometrically operable" (*20*) ionizations with acid dissociation constants of approximately 4.5 (carboxyl group?) and $\geqq 11.5$. In the presence of divalent metals, especially Cu^{++} and Co^{++}, its rate of decomposition to intermediate II is greatly decreased ($t_{1/2} \geqq$ 20 min), presumably by formation of metal complexes. Neither the *pK*'s nor the spectrophotometric characteristics of the compound appear to be affected by this complex formation. Intermediate II ($\lambda_{max} = 260$ mμ; $\varepsilon_{max} \simeq 10{,}000$) also shows two "spectrophotometrically operable" ionizations. The *pKa*'s are 5.5 and $\geqq 12$. The transition owing to the latter ionization ($\varepsilon_{max} \simeq 30{,}000$ at 260 mμ) appears to be irreversible and leads to the destruction of the compound. An analogous spectrophotometrically observable transition is brought about by the addition of certain metals (Co^{++}, Ni^{++}, or Cu^{++}, but not Fe^{++}, Mg^{++}, or Mn^{++})

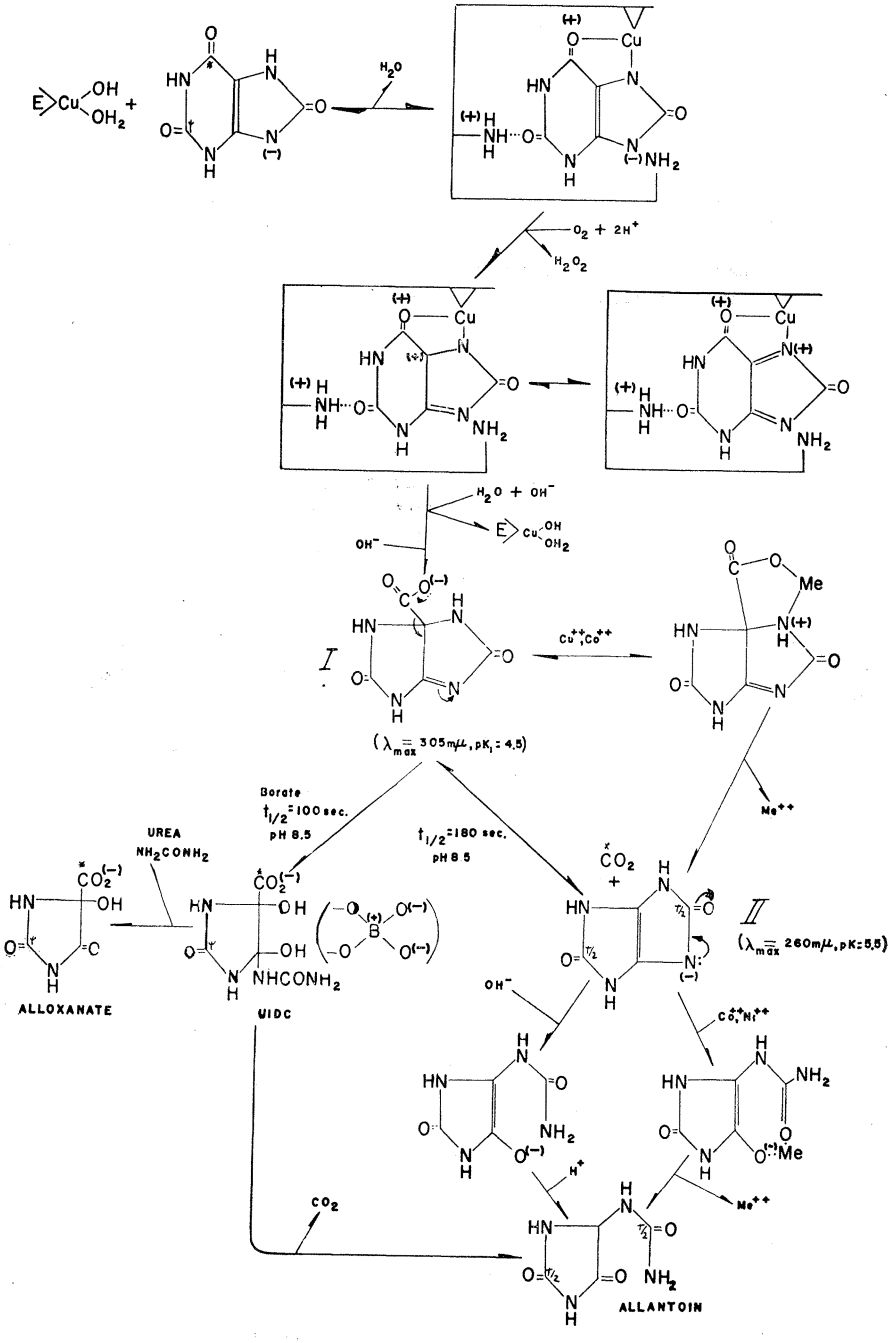
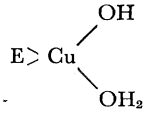


Fig. 1. Scheme representing transformations.

leading to the formation of reasonably stable metal chelates ($\lambda_{max} = 275$ mμ; $\varepsilon_{max} \simeq 30{,}000$).

Reactions Catalyzed by the Enzyme and Subsequent Chemical Events

It is believed that the scheme shown in Fig. 1, although it is speculative in certain instances, represents adequately the transformations described so far, and that it is based on reasonably firm experimental bases in the realms of enzyme chemistry, inorganic chemistry, and organic chemistry. Thus, the enzyme-copper complex is presented as



because, by analogy with the corresponding aqueo-complexes, this would appear to be the most reasonable structure in the *p*H range under consideration (*21*). It is also in accord with the *p*H dependences of the binding of urate and cyanide to the enzyme. The representation of the dehydrogenation reaction as leading from a carbanion to a carbonium ion is in accord with Bentley and Neuberger's postulate (*6, 18*), as are the ring-contraction reaction of the carbonium ion, a transformation amply documented in organic chemistry (*22*) (Wagner-Meerwein rearrangement), and the structure of intermediate I (Fig. 1). The reversible carboxylation reaction similarly is one not without precedent in organic chemistry (*23*), for it is analogous to the decarboxylations of β-ketonic acids (*24*). Numerous other compounds have been suggested as possible intermediates at one time or another. Of these, uric acid-5,6-glycol (*25*), 5-hydroxy-3,7-dioxo-2,4,6,8-tetraazabycyclo · (3,3,0) · octane-1-carboxylic acid (hydroxyacetylene diureido carboxylic acid, HDC) (*6, 18, 26*) and uroxanic acid (*27*) were prepared and tested under the reaction conditions used. They were all excluded as obligatory intermediates, since they were either stable or gave rise to products other than allantoin.

Mechanism of the Oxidation Reaction

The transfer of electrons from urate to oxygen as catalyzed by the enzyme presents an interesting problem. A possible mechanism would be the reversible reduction and reoxidation of the enzyme-bound copper. This was suggested as the most plausible mechanism for the analogous oxidations effected by other cuproenzymes (*28*). This mechanism is probably not operative as such in the present case. Since there is only one copper atom per enzyme molecule, and since the kinetic measurements definitely suggest that a single enzyme-urate complex is involved in the rate-limiting step, this would entail the removal of 1 electron at a time from urate (*29*)—that is, the transformations summarized in reaction 6 involving the semiquinoid free radical U.

$$U^- + Cu^{++}E \rightleftharpoons [U^-Cu^{++}E] \rightarrow [U \cdot Cu^{+}E] \quad (6a)$$

$$[U \cdot Cu^{+}E] + O_2 \rightarrow [U \cdot Cu^{++}E] + O_2^- \quad (6b)$$

$$[U \cdot Cu^{++}E] \rightarrow [U^+Cu^{+}E] \rightarrow U^+ + Cu^{+}E \quad (6c)$$

$$Cu^{+}E + O_2^- \rightarrow Cu^{++}E + O_2^= \quad (6d)$$

Unless the affinity of the free radical for the enzyme is exceedingly great, one would expect the formation of rearrangement and polymerization (that is, dimers) products of urate. No evidence for such products has ever been obtained in the reaction under consideration. Another possible mechanism is one discovered by Chance for the hydroperoxidases (*30*) and suggested as being applicable to a variety of other dehydrogenation reactions as well (*31*).

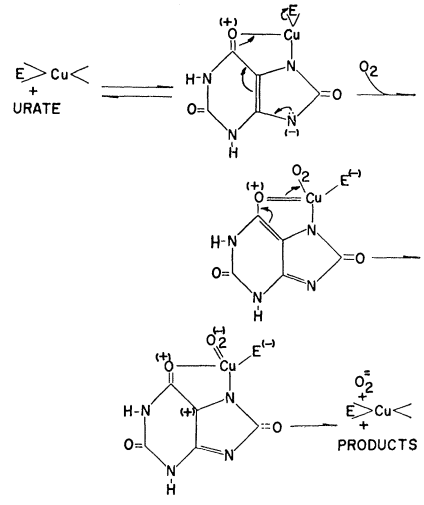
$$U^- + Cu^{++}E \rightleftharpoons [U^-Cu^{++}E] \quad (7a)$$

$$2H^+ + [U^-Cu^{++}E] + O_2 \rightarrow [U^+Cu^{++}E] + H_2O_2 \quad (7b)$$

$$[U^+Cu^{++}E] \rightleftharpoons U^+ + Cu^{++}E \quad (7c)$$

This mechanism has as a consequence the direct proportionality of K_S, the Michaelis constant for the substrate (urate), to the acceptor (O_2) concentration. This question has been investigated, and no such dependence has been found. As a matter of fact the K_S in pure oxygen was lower than the corresponding constant in air.

All the experiments on the kinetics of the oxidation of the only two substrates acted on by the enzyme—urate and 2,6-dioxy-8-aminopurine (the latter being oxidized at about 10^{-3} the rate of the former)—are consistent with the hypothesis that the actual electron transfer occurs within a ternary complex of substrate, enzyme, and acceptor. A possible elaboration of this hypothesis is presented in the following reaction.



The metal is probably in the oxidized form in the catalytically active complex. Substances that are capable of both complexing and reducing the copper from the cupric to the cuprous form are effec-

Table 1. Kinetic constants (*14*) of highly purified uricase (*8*) [0.01*M* tris(hydroxymethyl)aminomethane buffer at 20°C] and acid dissociation constants of binding sites on enzyme and enzyme-substrate complex.

Constant	Value
Kinetic constants	
$V'_{max} = k_3[Et]$ (*p*H-independent maximal velocity)	1.7×10^{-2} μmole × min^{-1} × μg^{-1} of enzyme
k_3 (assuming a molecular weight of 100,000)	$1.7 \times 10^3 \times min^{-1}$
K_{ES} (*p*H-independent Michaelis constant)	$1.7 \times 10^{-5} M$
K_{SS} (excess substrate inhibitor dissociation constant)	$4.0 \times 10^{-4} M$
ΔE (activation energy)	1.24×10^4 cal × $mole^{-1}$
Acid dissociation constants	
pK_{aE}	7.5
pK_{bE}	9.2
pK_{aES}	7.2
pK_{bES}	9.5

Table 2. Enzyme-inhibitor dissociation constants for uricase-2,6,8 trisubstituted purine complexes (all in 0.01*M* tris-borate at *p*H 8.5).

Substituent in position			K_I (*M*)
2	6	8	
—Cl	—Cl	—Cl	8.0×10^{-7}
—Cl	—Cl	—OH	1.3×10^{-6}
—OH	$—NH_2$	$—NH_2$	1.8×10^{-6}
—OH	—OH	—H	1.2×10^{-5}
—OH	—OH	—OH	2.3×10^{-5}
—OH	—OH	—OH(urate)	2.5×10^{-5} (K_S at *p*H 8.5)
—Cl	$—NH_2$	—OH	4.0×10^{-5}
—OH	$—NH_2$	—OH	1.5×10^{-4}
$—NH_2$	$—NH_2$	—OH	5×10^{-4}
$—NH_2$	—OH	$—NH_2$	0
$—NH_2$	$—NH_2$	$—NH_2$	0

tive inhibitors for the enzyme (*7*). Williams has accounted for the great catalytic efficiency of cupric chelates on theoretical grounds (*32*). He postulates the *d*-orbitals of divalent metal ions as forming strong, continuous, overlapping molecular orbitals with the π- and σ-orbitals of certain ligands. We may extend this picture to include the substrate and acceptor in the present case. Electrons, either singly or in pairs, may then be capable of being transferred directly from the former to the latter. The metal is pictured as (i) providing a locus of physical attachment for both moieties of the catalytic complex, (ii) polarizing the electrons, to be transferred, away from the substrate by virtue of its strong electrophilic character, and (iii) permitting the actual interpenetrations of orbitals which we have alluded to. The somewhat greater liability to dissociation from the protein of the singly linked copper within the ternary complex may provide an explanation of two phenomena previously observed with other copper proteins—the exchange of radioactive copper with the copper of ascorbate oxidase, which occurs only in the presence of substrate and oxygen simultaneously (*33*), and the "reaction inactivation" common to many different cuproenzymes (*28*).

A somewhat similar picture has already been proposed to account for a large number of observations in the metalloflavoprotein series (*34*). Its possible extension to other metallodehydrogenases, such as other cuproenzymes and the zinc-pyridino-proteins, is now under investigation in this laboratory.

This investigation was supported by grants-in-aid by the American Cancer Society (on recommendation by the Committee on Growth, National Research Council) and by the National Institutes of Health, U.S. Public Health Service, to one of us (H. R. M.).

References and Notes

1. W. Wiechowski, *Beitr. chem. physiol. Pathol.* 9, 295 (1907); F. Batelli and L. Stern, *Biochem. Z.* 19, 219 (1909).
2. D. Keilin and E. F. Hartree, *Proc. Roy. Soc. London* B119, 114 (1938).
3. J. N. Davidson, *Biochem. J. London* 32, 1326 (1938); *ibid.* 36, 252 (1942); C. G. Holmberg, *ibid.* 33, 1901 (1939); K. I. Altman, K. Smull, E. S. G. Barron, *Arch. Biochem.* 21, 153 (1949); E. Leone, *Biochem J. London* 54, 353 (1953).
4. F. Felix, F. Scheel, W. Schuler, *Hoppe-Seyler's Z. physiol. Chem.* 180, 90 (1929); W. Schuler, *ibid.* 208, 237 (1932).
5. E. Praetorius, *Biochim. et Biophys. Acta* 2, 602 (1948).
6. R. Bentley and A. Neuberger, *Biochem. J. London* 52, 694 (1952).
7. H. R. Mahler, G. Hubscher, H. Baum, *J. Biol. Chem.* 216, 625 (1955).
8. H. Baum, G. Hubscher, H. R. Mahler, *Biochim. et Biophys. Acta,* in press; G. Hubscher, H. Baum, H. R. Mahler, *ibid.,* in press.
9. We wish to express our sincere appreciation to David E. Green, director of the Institute for Enzyme Research, for his encouragement of this investigation. It was supported by grants-in-aid by the American Cancer Society (on recommendation by the Committee on Growth, National Research Council) and by the National Institutes of Health, U.S. Public Health Service, to one of us (H. R. M.).
10. F. Kubowitz, *Biochem. Z.* 292, 221 (1937); 299, 32 (1938).
11. A. Albert, *Biochem. J. London* 54, 646 (1953).
12. I. M. Klotz, in *The Mechanism of Enzyme Action,* W. D. McElroy and B. Glass, Eds., (Johns Hopkins Press, Baltimore, Md., 1954), p. 257.
13. A. E. Martell and M. Calvin, *Chemistry of the Metal Chelate Compounds* (Prentice-Hall, New York, 1952); R. J. P. Williams, *J. Phys. Chem.* 58, 121 (1954).
14. S. G. Waley, *Biochim. et Biophys. Acta* 10, 27 (1953); M. Dixon, *Biochem. J. London* 55, 161 (1953); V. Massey and R. A. Alberty, *Biochim. et Biophys. Acta* 13, 354 (1954); C. Frieden and R. A. Alberty, *J. Biol. Chem.* 212, 859 (1955); K. J. Laidler, *Trans. Faraday Soc.* 51, 528, 540, 550 (1955).
15. J. Schubert, in *Chemical Specificity in Biological Interactions,* R. N. Gurd, Ed. (Academic, New York, 1954).
16. D. Keilin and E. F. Hartree, *Proc. Roy. Soc. London* B119, 141 (1936).
17. We are indebted to P. P. Cohen for generous gifts of the 2-C^{14} and 8-C^{14} uric acids and to A. Neuberger for his generous gift of C^{14}-diaminouracil sulfate that was used as the precursor of 6-C^{14} uric acid (*18*). The substituted purines were kindly provided by G. B. Brown.
18. C. E. Dalgliesh and A. Neuberger, *J. Chem. Soc.* 1954, 3407 (1954).
19. E. S. Cannelakis and P. P. Cohen, *J. Biol. Chem.* 213, 385 (1955).
20. F. Bergmann and S. Dikstein, *J. Am. Chem. Soc.* 77, 691 (1955); L. F. Cavalieri *et al., ibid.* 76, 1119 (1954); D. Shugar and J. J. Fox, *Biochim. et Biophys. Acta* 9, 199 (1952).
21. J. Bjerrum, *Metal Amine Formation in Aqueous Solution* (Haase, Copenhagen, Denmark, 1941).
22. N. J. Leonard and W. C. Wildman, *J. Am. Chem. Soc.* 71, 3089 (1949); N. J. Leonard and W. V. Ruyle, *ibid.* 71, 3094 (1949).
23. F. Brown and R. Hammick, *J. Chem. Soc.* 1949, 659 (1949).
24. B. R. Brown, *Quart. Revs. London* 5, 131 (1951); J. Hine, *Physical Organic Chemistry* (McGraw-Hill, New York–London, 1956), pp. 283–291.
25. H. Biltz, *Ber. deut. chem. Ges.* 47, 460 (1914).
26. R. Behrend, *Ann. Chem. Justus Liebigs* 333, 153 (1904); W. Schuler and W. Reindel, *Hoppe-Seyler's Z. physiol. Chem.* 208, 248 (1932); *ibid.* 215, 258 (1933).
27. F. W. Klemperer, *J. Biol. Chem.* 160, 111 (1945).
28. See, for example, the discussion by T. P. Singer and E. Kearney in *The Proteins,* H. Neurath and K. Bailey, Eds. (Academic, New York, 1954), vol. II-A.
29. L. Michaelis, in *The Enzymes,* J. B. Sumner and K. Myrback, Eds. (Academic, New York, 1952), vol. II-1.
30. B. Chance, in *Techniques in Organic Chemistry,* S. L. Friess and A. Weissberger, Eds. (Interscience, New York, 1953), vol. VII.
31. B. Chance and A. M. Pappenheimer, Jr., *J. Biol. Chem.* 209, 931 (1954); H. Theorell and B. Chance, *Acta Chem. Scand.* 5, 1127 (1951).
32. R. J. P. Williams, *Nature* 177, 304 (1956).
33. M. Joselow and C. R. Dawson, *J. Biol. Chem.* 191, 1, 11 (1951).
34. H. R. Mahler and J. Glenn, in *Inorganic Nitrogen Metabolism: Function of Flavoproteins,* W. D. McElroy and B. Glass, Eds. (Johns Hopkins Press, Baltimore, Md., 1956).

Amedeo Avogadro

Two thousand years ago Lucretius in Rome expounded the doctrine of atoms. He expressed in immortal language the speculations of the Greek philosophers, and he described with the vividness of a great poet the movements, the unions and separations of the tiny corpuscles of which he conceived all things to be composed. The atoms had many qualities which modern science assumes even today. Vigorous motion under the appearance of rest, penetration of heat and cold depending on this movement, hooks for attachment to others, and even an unpredictable "clinamen" or swerve, which is a sort of fantastic anticipation of the uncertainty principle of quantum mechanics.

This was magnificent and represented a wonderful intuitive insight into the working of nature, but it was not science. There was no link with quantitative experimentation, the construction of which connection was lacking until modern times. Then, one might say suddenly, the science of chemistry was created by the vision of a few great men, among whom was Amedeo Avogadro.

The recognition by Avogadro of the distinction between atoms and molecules was the key which opened the treasury of structural chemistry: a treasury whose riches are not yet exhausted. The establishment of the true doctrine about the nature of the particles of the elementary gases rendered possible the development of the kinetic theory and the understanding of the energy relationships of these particles.

On this basis was founded the study not only of the structure of substances but of the functional relationships that govern chemical change: chemical kinetics. A true doctrine of molecules was the necessary precursor of what may

This article, by Cyril Hinshelwood, and the following one, by Linus Pauling, are based on addresses given in Rome on 6 June, when the Accademia Nazionale dei XL commemorated the centenary of the death of Avogadro. At the ceremony, a new medal, commemorating Avogadro, was presented by the Accademia, for the first time, to Sir Cyril and to Dr. Pauling.