

iodination of tyrosine residues of protein A leads to significant loss of functional activity. Although enzyme-labelled protein A appears to perform better than enzyme-labelled anti-IgG reagents, none of the studies on the use of enzyme-labelled protein A to detect various antibodies reports the stoichiometry of the conjugate, kinetic parameters or optimum conditions which are essential for its use in a reproducible and quantitative enzyme immunoassay. The inherent problem of synthesizing enzyme-antibody (antigen) conjugates of fixed stoichiometry separately for quantitation of each antigen (antibody) by enzyme immunoassay has recently been obviated by the use of protein A-enzyme monoconjugate^{16,17}. Protein A-enzyme monoconjugates, prepared on an affinity matrix or by using a heterobifunctional reagent, were used to quantitate antigens, haptens, antibodies and biologically important receptors¹⁸. In each case kinetic parameters and optimum conditions have been established. Recently, the problem of prior coupling of an enzyme label to antigen, antibody or hapten concerned has also been overcome in non-covalent enzyme labelling by the 'protein A bridge' between the primary antibody and soluble peroxidase-antiperoxidase complex, and the method has the potential for both antigens and haptens.

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50 Years Ago

The discovery of the ornithine cycle of urea synthesis

Hans A. Krebs

The discovery of the ornithine cycle of urea synthesis in 1932¹, provided the first indication that some metabolic pathways are arranged in a cyclic manner. I started to study urea synthesis in 1931, in my thirty-first year when, for the first time, I was completely free to choose my own field of research. Until then I had served a long apprenticeship. This began with the study of medicine from 1919 to 1924, continued with a course in chemistry in 1925, and was completed by an assistantship in the laboratory of Otto Warburg in Berlin from 1926 to 1930.

When I left Warburg's laboratory I decided to return to clinical medicine, in the hope that I might be able to combine scientific research with clinical work. I was lucky to find a suitable position as a clinical assistant at the Altona (today part of Hamburg) Municipal Hospital under Professor Leo Lichtwitz who himself was keenly interested in the application of biochemistry to clinical problems. He was the author of an important book, *Klinische Chemie*, first published in 1913. This hospital, though of very high standing, was not connected with a university, which was a disadvantage. In 1931, I moved to the University of Freiburg im Breisgau, where Professor S. J. Tannhauser of the Department of Medicine, accepted me as one of the 'assistants'.

It was only at this stage that I became free

in the choice of my research topic. When I left Warburg's laboratory, he agreed to support an application for research equipment – mainly the Warburg and the Van Slyke manometric apparatus – on the understanding that I would study a topic of his choice, namely the proteolytic capacity of tumour tissue. Having demonstrated that tumour tissue is distinguished from most other tissues by a high glycolytic capacity, Warburg thought it useful to compare the proteolytic potential of animal tissues. However, the work showed that malignant tumour tissue did not differ strikingly in this respect from normal tissues.

The basic idea behind my choice of a problem was a simple one. In Warburg's laboratory I had seen the value of the tissue-slice technique in studying the respiration and glycolysis of animal tissues under controllable conditions *in vitro*. Before then it had not been possible to study the respiration and glycolysis of animal tissues (except blood cells) satisfactorily *in vitro* because the earlier tissue preparations – chopped and minced tissue – had lost most of their respiratory and glycolysing activity. Warburg had used the slice technique solely for measuring the overall rate of the degradative reactions which supply energy. My idea was to use slices for the study of other aspects of metabolic processes. I especially had in mind synthetic reactions, and I hoped that slices would also lend themselves to the study of the intermediary metabolism of respiration. When I mentioned these ideas to Warburg he showed no interest. He did not encourage work which was not directly related to his own field.

I chose as a first subject the study of the synthesis of urea in the liver, because it appeared to be one of the simplest biosynthetic processes and one that occurs at a high rate. Since all previous attempts to

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obtain a synthesis of urea in isolated liver preparations – other than the perfused liver – had been unsuccessful, I thought it necessary, as a preliminary, to design a medium which simulated blood plasma as closely as possible in respect to the inorganic constituents. The saline media of Ringer, Locke and Tyrode were grossly deficient in the major plasma constituents – especially bicarbonate and CO_2 . Warburg's medium included bicarbonate and CO_2 , but lacked magnesium, phosphate and sulphate; and the concentration of other ions would have been closer to the physiological range.

The idea behind the design of a new saline medium was the conviction that the ionic concentrations in blood plasma (which are almost identical in all mammalian species and very similar in other vertebrates) are not accidental, but have evolved to become optimally attuned to the functions of the various organs. It was already well established that many organs need the correct ionic environment to function properly. The new medium, which included magnesium, phosphate and sulphate at physiological concentrations, has proved decisively superior to all earlier saline plasma substitutes. This medium still differs from plasma in respect to the colloid-osmotic pressure and the organic constituents of plasma such as glucose, fatty acids, amino acids and hormones. The colloid-osmotic pressure can be adjusted by the addition of bovine serum albumin, and other substances can easily be added; but isotonicity must be maintained.

Before testing the capacity of liver slices to synthesize urea, I thought it necessary to devise a quick, sensitive and specific method for the determination of urea, because Warburg had conveyed to me the importance of carrying out large numbers of experiments when exploring unknown territories. So I adapted the urease method of Marshall^{2,3} and Van Slyke⁴ to manometry. A solution of urease (pH 5.0) was added to the solution to be examined and the CO_2 evolved was measured. A dozen determinations could be carried out in less than 1 h. Quantities of approximately 0.05 mg could be determined with an accuracy of a few per cent. Incidentally, the principle of the urease method, introduced by Marshall in 1913, was one of the earliest analytical procedures employing enzymes as analytical tools.

Plan of work

Preliminary tests with tissue slices showed that liver slices synthesize urea at rates expected from what was known for the intact body, and that the rates were greatly raised by the addition of ammonium salts or certain amino acids. After this hopeful start

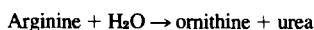
I decided to measure systematically the rate of urea synthesis in the presence of a variety of precursors, hoping that the results might throw light on the chemical mechanism of urea synthesis. I had no preconceived idea and no hypothesis about this mechanism but I had in mind Warburg's work on cell respiration in which he had studied the rate of respiration in the presence of cyanide, carbon monoxide, narcotics and other substances, and succeeded in drawing far-reaching conclusions about the properties of the enzymes of respiration. In this work I was joined by a medical student, Kurt Henseleit, who wished to do a piece of research for his MD thesis. He proved a very skilful experimenter who rapidly acquired the necessary laboratory techniques.

Questions which suggested themselves for investigation included the following:

- (1) Is ammonia an obligatory intermediate in the conversion of amino nitrogen to urea nitrogen? If it is, ammonia must yield urea at least as rapidly as amino acids. If it is not, the rate of urea formation from amino acids might be more rapid than the rates from ammonia. (It became known more than 20 years later that half of the urea nitrogen can be derived directly from amino acids, without passing through the stage of ammonia.)
- (2) How do the rates of urea formation from various amino acids compare?
- (3) Do substances which had been suspected to be intermediates, for example, cyanate, behave like intermediates in that they can be conveyed into urea?
- (4) Do pyrimidines yield urea directly or via ammonia?

The first crucial finding

We measured the rate of urea synthesis under many different conditions, including the presence of mixtures of ammonium ions and amino acids. In the course of these experiments we discovered exceptionally high rates of urea synthesis when both ornithine and ammonium ions were present. The significance of this finding was not at once obvious. It took a full month to find the correct interpretation. At first we were sceptical about the observations. Was the ornithine perhaps contaminated with arginine? The answer was no. Then it occurred to us that the effect of ornithine might be related to the presence of arginase in the liver, the enzyme which converts arginine into ornithine and urea, as shown by Kossell and Dakin^{5,6} in 1904:



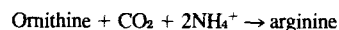
Arginase was known to be highly active in the liver of animals (mammals, amphibians and certain reptiles) which can synthesize urea from ammonia and amino acids⁷. The

coincidence of the exceptionally high activity of this enzyme and the occurrence of urea synthesis in the liver raised the suspicion of a connection, but for some weeks we were unable to see one, nor had this occurred to any previous investigator. The solution to the problem developed gradually as we studied the ornithine effect in detail.

The analysis of the ornithine effect

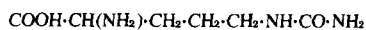
In the first experiments which revealed the ornithine effect, we used high concentrations of ornithine because we wanted to find out if ornithine can act as a nitrogen donor. When lower ornithine concentrations were tested, the stimulating effect remained. Finally it became clear that one molecule of ornithine can bring about an extra formation of over 20 molecules of urea, provided that ammonia was present. Moreover, the amino nitrogen content of the medium, as measured by the Van Slyke technique, did not decrease during the synthesis of urea, and the total urea nitrogen could be accounted for by the disappearance of ammonia. This established that ornithine acts like a catalyst; it is not used up, and there is no simple stoichiometry between the amount of ornithine present and its effect on the rate of urea production.

When considering the mechanism of this catalytic action, I was guided by the idea that a catalyst must take part in the reaction and form intermediates. The reactions of the intermediates must eventually regenerate ornithine and form urea. Once these postulates had been formulated, it became obvious that arginine fulfilled the requirements of an expected intermediate. This meant that a formation of arginine from ornithine had to be postulated according to the overall equation



Search for intermediates

It was also at once obvious that the synthesis of arginine from ornithine must involve more than one step, since four molecules – one ornithine, one CO_2 , two NH_3 – had to interact. So I began to search for possible intermediates between ornithine and arginine. Chemical considerations suggested that citrulline



might be an intermediate. This substance had just been identified independently by two biochemists in entirely different contexts. Wada⁸ isolated it, in 1930, from watermelons (*Citrullus*), and, in 1931, Ackermann⁹ isolated it as a product of the bacterial degradation of arginine. I wrote to both and obtained a few milligrams from

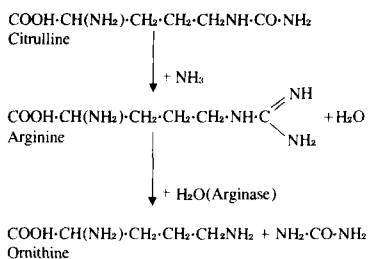


Fig. 1.

each, sufficient to do the decisive tests. These entirely fulfilled expectations. Urea is formed rapidly in the presence of citrulline and ammonium salts as shown in Fig. 1.

It then became possible to formulate a cyclic process for urea formation from carbon dioxide and ammonia, with citrulline and arginine as intermediate stages (Fig. 2).

The ornithine cycle as a new pattern of metabolic organization

No cycle of this kind (a 'metabolic cycle') in which low molecular intermediates are formed cyclically, had been known before although different kinds of cycles were familiar to biologists and chemists. There are biological cycles like the menstrual cycle and the diurnal cycle. Meyerhof and Cori spoke of a lactic acid cycle in muscle and liver. Lactate is produced from glycogen under anaerobic conditions during exercise and resynthesized aerobically to glycogen. None of these cycles is strictly analogous to the ornithine cycle, but subsequently a large number of metabolic cycles, exactly analogous to the ornithine cycle, have been discovered. Thus the ornithine cycle revealed a new pattern of the organization of metabolic processes. It is now clear why so many metabolic processes are arranged in a cyclic manner. In many situations cyclic processes are more efficient than any feasible alternative and, therefore, they have survived in the course of evolution.

Later elaborations of the cycle

The experiments of 1931 and 1932, established the outlines of the pathway by

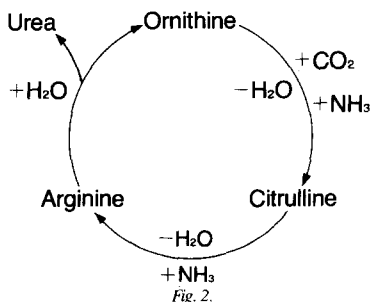


Fig. 2.

which urea is synthesized in the liver. We carried out a few experiments on urea synthesis in ground-up liver, but all these experiments were negative. In retrospect we now appreciate that the time was not ripe for unravelling details of the enzymic mechanism. The history of the development of the ornithine cycle illustrates the general experience that at any one time the solution of a problem can be advanced only to a limited extent. Soon seemingly impenetrable walls obstruct progress. After a time, however, advances in collateral fields overcome the barriers – sometimes by circumventing them, sometimes by demolishing them piece by piece. Where I had failed, Cohen and Hayano succeeded 14 years later. By that time much had been learned about the preservation of complex metabolic processes in cell-free homogenates.

Milestones in the further elaboration of the cycle were the discovery of carbamoyl phosphate, the identification of argininosuccinate as an intermediate in the formation of arginine from citrulline, the discovery of the regulatory role of *N*-acetyl glutamate in the activity of carbamoyl phosphate synthetase and characterization of all the enzymes involved. These developments are associated with the names of Philip Cohen and his associates, Sarah Ratner, Fritz Lipmann, Mary Ellen Jones and Santiago Grisolia. Their work and that of others has been summarized in the book *The Urea Cycle*¹⁰.

Some reactions of the scientific community

The great majority of my fellow scientists accepted the evidence presented in 1932 as convincing and commented enthusiastically. Warburg arranged an invitation for me to speak at the Kaiser Wilhelm Gesellschaft, Meyerhof invited me to lecture at Heidelberg, and Hopkins referred to it in his Presidential Address at the Royal Society. Knoop, to whom I submitted the paper for publication in his capacity as an editor of the *Zeitschrift für physiologische Chemie*, commented in a very complimentary way and added that he felt really stupid that it had never occurred to him before that arginase might play a role in the synthesis of urea.

But there were also adverse criticisms. In 1934, E. S. London, of Leningrad, argued that ornithine had no effect on the isolated perfused dog liver^{11,12}. In 1942 Trowell, of the Cambridge Physiological Laboratory, reported that the perfused rat liver did not respond to ornithine¹³. At the same time Bach and Williamson, of the Cambridge Biochemical Laboratory, claimed to have shown that dog liver can form urea from

ammonia even when arginase is completely inhibited by high concentrations of ornithine, and they concluded that liver can synthesize urea without participation of arginase¹⁴. As late as 1956, Bronk and Fisher reported from my own department at Oxford that under certain conditions citrulline is less effective than ornithine in promoting urea formation from ammonia, and they concluded that citrulline is not necessarily an intermediate in the conversion of ornithine to arginine¹⁵. The results of London and of Trowell were due to an inadequate perfusion technique. The conclusions of Bach and Williamson were based on the wrong assumption that arginine and ornithine readily penetrate liver slices. In fact, the rates of penetration are relatively slow. Although the observations of Bronk and Fisher were correct, their interpretation was mistaken. They expected the kinetics of an homogeneous solution in the highly compartmented system of living cells. Permeability barriers, often unpredictable, can interfere and may cause deviations, usually of a minor quantitative kind, from a postulated kinetic behaviour. At that time, in 1956, the importance of permeability barriers at the plasma membrane and mitochondrial membrane was not sufficiently appreciated. Experience shows that to postulate, as Bronk and Fisher did, a second mechanism on the basis of some kinetic discrepancies is far more rash and fanciful than to accept kinetic abnormalities on account of permeability barriers and other complications when dealing with very complex systems.

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