

THE DISCOVERY OF THE ORNITHINE CYCLE OF UREA SYNTHESIS

The Tissue Slice Technique

In the laboratory of Otto Warburg where I worked from 1926 to 1930 and served my apprenticeship in biochemistry, I became impressed by the achievements and potentialities of the tissue slice technique. Warburg had introduced this technique in 1923 in order to study the metabolism of cancer cells (1). In 1908 he had discovered the great increase, about six fold, in respiration which occurs when the sea urchin egg is fertilised and begins to grow (2). After his return from military service in World War I, he intended to investigate whether there is a similar rise in the oxygen consumption when normal body cells begin to grow and become cancerous. As a first step he had to develop a reliable method for the measurement of cell respiration as no suitable technique was available, and this led to the development of manometry and to procedures for handling isolated tissues *in vitro* under precisely controlled conditions. In earlier work Warburg had found that cell respiration is liable to disappear on mincing a tissue and suspending it in a saline medium (3). From this observation he drew the conclusion that cell respiration is dependent on the intactness of cell structure. Slices, being 0.2 to 0.4 mm thick, contain mainly intact cells and are thin enough to permit adequate oxygenation and nutrient supply by diffusion.

Using the slice technique, Warburg found that respiration of cancer cells is no greater than that of nongrowing body cells but that there is a second aerobic source of energy – glycolysis – which, though not specific for cancer, is universally found to occur at high rates in neoplastic cells. The comparison of cancer cells and other tissues led to many other important new observations. Warburg was first to measure the normal rates of oxygen consumption and lactic

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acid production of a great variety of animal tissues. Among the new observations was the high rate of the aerobic glycolysis of the retina of warm-blooded animals.

Warburg used the tissue slice technique only for the study of degradative metabolic processes – respiration and glycolysis. It occurred to me that slices might also be able to make use of the energy which they derive from respiration and fermentation, to bring about syntheses, i.e. energy-consuming reactions. If this proved correct, an entirely new experimental approach to metabolic problems would be open. The old established alternative methods, such as experiments on the intact organism or the perfusion of isolated organs had, at that time, great limitations.

The Choice of the Problem

In testing the idea that slices might perform biosyntheses, I chose what seemed to me one of the simplest biosyntheses, the synthesis of urea by the mammalian liver. This process seemed to be particularly suitable because it has the advantage that its rate *in vivo* can be very high and if the *in vivo* rates occurred in slices they would be easily measurable. A human adult on an ordinary diet, for example, produces about 30 g of urea in 24 h, and on a protein-rich diet it can be three times greater. Such rates, 1.5 mole per day, are not equalled by any other net biosynthesis in the body. Perhaps the next most rapid biosynthesis is gluconeogenesis which may amount to 100 g of glucose, or 0.55 mole, in 24 h.

There are of course higher rates of synthesis of intermediates, as opposed to net products. Thus the amounts of ATP synthesised in



Dr Kurt Henseleit and Sir Hans Krebs photographed in Germany in 1972 – forty years after their joint work on urea synthesis.

the adult human body is of the order of 180 mole per day on a calorie consumption of 3,000 per day, but unlike urea, ATP does not accumulate. Since it is used up as rapidly as it is formed, its steady state concentration in the tissues is very low: at any time there is probably less than 0.1 mole of ATP in the adult human body.

Before testing the capacity of liver slices to synthesise urea, I thought it necessary to devise a quick, sensitive and specific method for the determination of urea, and I adapted the urease method of Marshall (4), and Van Slyke (5), to manometry. A solution of urease is added at pH 5.0 to the solution to be examined and the CO₂ evolved is measured (6). This procedure permits a dozen determinations to be carried out within less than one hour. Quantities of approximately 0.05 mg can be determined with an accuracy of a few per cent. The method is still useful today. Incidentally, the principle of the urease method, introduced by Marshall in 1913, was one of the earliest analytical procedures employing enzymes as analytical tools. Now there are many hundreds of enzymic analytic methods.

Design of a New Saline Medium

Whether slices could bring about synthetic processes was very uncertain, and in order to provide optimum conditions for the survival of tissue slices, I tried to design a medium which simulated blood plasma as closely as possible in respect to the inorganic constituents. This meant modifications of the type of saline solutions which Ringer (7), Locke (8), Tyrode (9) and Warburg (10) had introduced. The media of Ringer, Locke and Tyrode were grossly deficient in two of the major plasma constituents – bicarbonate and CO₂. They were arrived at mainly on the basis of trial and error, especially when testing for a medium which maintained the activity of the frog heart. Warburg included bicarbonate and CO₂ but his medium lacked magnesium, phosphate and sulphate, and the concentration of other ions was not as close to the physiological range as is possible. The idea behind the design of the new saline 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 be optimally attuned to the functions of the various organs. At that time it was already well established that the function of many organs depends on the composition of the ionic environment. The new medium which included magnesium, phosphate and sulphate in physiological concentrations has subsequently proved decisively superior to all earlier saline plasma substitutes, not only in biochemical, but also in physiological and pharmacological work. It is now widely used.

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 commercial bovine serum albumin, and other substances can easily be added but attention must be paid to maintaining isotonicity.

Plan of Work

Preliminary tests with tissue slices showed that liver slices synthesise urea at rates expected from what was known for the intact body, and 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 may 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 had asked my chief, Prof. Thannhauser, to suggest a subject for his M.D. thesis. Thannhauser referred him to me. 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, e.g. cyanate, behave like intermediates in that they can be converted into urea?
4. Do pyrimidines yield urea directly, or via ammonia?

The First Crucial Finding

So we measured the rate of urea synthesis under many different conditions, and these included the presence of mixtures of ammonium ions and amino acids (6). It was in the course of these experiments that we discovered the exceptionally high rates of urea synthesis when both ornithine and ammonium ions were present. The interpretation 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 correctness of 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, known since the work of Kossel & Dakin (11) in 1904:

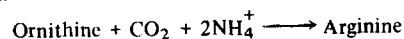


Arginase was known to occur in very high activity in the liver of those animals (mammals, amphibians and certain reptiles) which can synthesise urea from ammonia and amino acids (12,13). The coincidence of the exceptionally high activity of this enzyme and the occurrence of urea synthesis in the liver raised the suspicion of a connection but for some weeks we were unable to visualize one, nor had this occurred to any previous investigator. The solution of the problem developed gradually as the ornithine effect was studied in detail.

The Analysis of the Ornithine Effect

In the first experiments which revealed the ornithine effect, the concentration of ornithine had been high because it had been the intention to explore whether ornithine can act as a nitrogen donor. When lower ornithine concentrations were tested, the stimulating effect remained and the final result of this aspect of the work was the discovery 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 did not decrease during the synthesis of urea and the total urea nitrogen could be accounted for by the disappearance of ammonia. This established the fact 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 upon the rate of urea production.

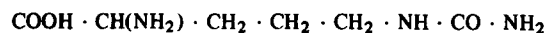
When considering the mechanism of this catalytic action I was guided by the idea that a catalyst must take part in the reaction and form intermediates. The reactions of the intermediates must eventually regenerate ornithine and form urea. Once these postulates have been formulated it became obvious that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



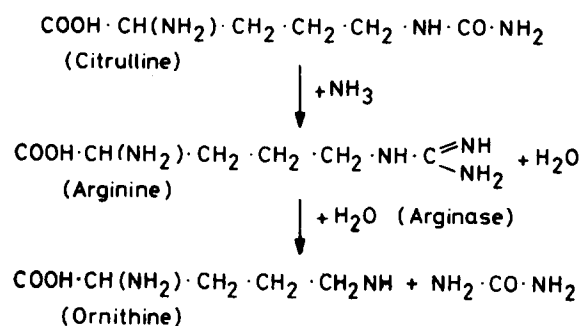
Search for Intermediates

It also became obvious at once that the synthesis of arginine from ornithine must involve more than one step since four molecules –

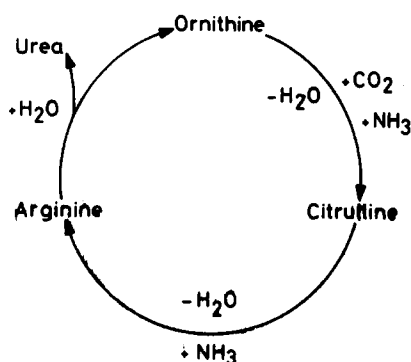
one ornithine, one CO_2 , two NH_3 – had to interact. So I began to search for possible intermediates between ornithine and arginine. Paper chemistry suggested that citrulline:



might play a role as an intermediate. This substance had just been identified independently by two biochemists in entirely different contexts. Wada (14) isolated it from water melons (*Citrullus*) and Ackermann (15) isolated it as a product of the bacterial degradation of arginine. I wrote to both and obtained a few milligrammes from each, sufficient to do the decisive tests. These entirely fulfilled expectations: they demonstrated the rapid formation of urea in the presence of citrulline and ammonium salts in accordance with this scheme:



On the basis of these findings it became possible to formulate a cyclic process of urea formation from carbon dioxide and ammonia, with citrulline and arginine as intermediate stages as shown below:—



The Ornithine Cycle as a Pattern of Metabolic Organisation

No cycle of this kind – which may be called “metabolic cycle” – had been known before, a cycle in which low molecular intermediates are formed cyclically. Entirely different kinds of cycles of course were familiar to biologists and chemists. There are the biological cycles like the menstrual cycle and the diurnal cycle. There is the carbon cycle involving photosynthesis in plants and combustion in animals. There is the life cycle of metamorphosing insects. There is the cell generation cycle. Closest to the metabolic cycles are the catalytic cycles of chemistry, for instance the actions of heavy metals in catalysing the knall-gas reaction. The combination of hydrogen and oxygen to form water is catalysed by platinum or palladium and this catalysis involves the intermediate formation of the hydride of the catalyst.

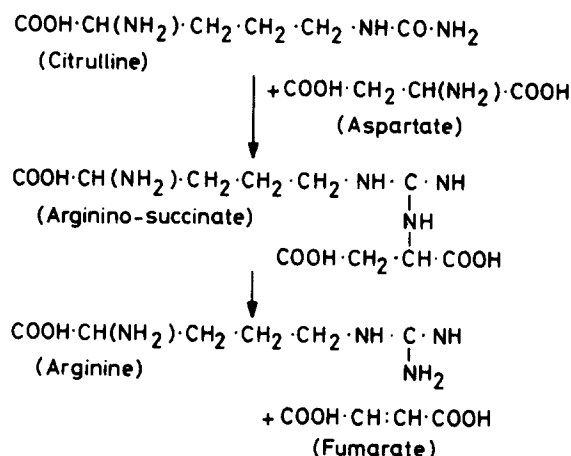
Meyerhof and Cori spoke of a lactic acid cycle in muscle and liver. Lactate is produced from glycogen under anaerobic conditions in exercise and resynthesised to glycogen. None of these cycles is strictly analogous to the ornithine cycle but subsequently a large

number of metabolic cycles, exactly analogous to the ornithine cycle, have been discovered. Thus the ornithine cycle revealed a new pattern of the organisation of metabolic processes.

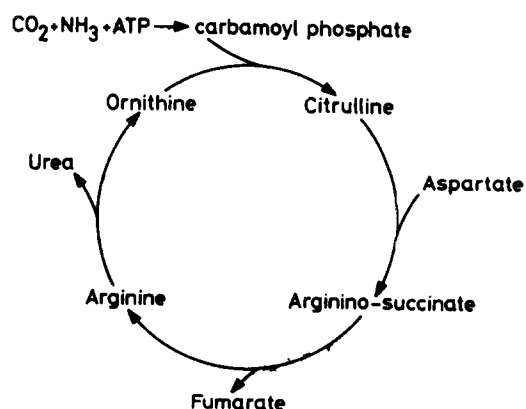
Later Elaborations of the Cycle

The work carried out in 1931 and 1932 established the outlines of the pathway by which urea is synthesised in the liver. The time then did not seem ripe for attempts to unravel the enzymic mechanisms involved. A first step in that direction were experiments aimed at obtaining a synthesis of urea in cell-free material but such experiments were negative in my hands. Where I had failed Cohen & Hayano (16) succeeded 14 years later. By that time much had been learned in general about how to preserve complex metabolic processes in cell-free homogenates and in tissue fractions. The development of the techniques for preparing metabolically active cell-free homogenates was a lengthy endeavour in which many biochemists took part. One of the chief requirements of homogenates turned out to be the need for added cofactors, such as pyridine nucleotides and ATP. The concentration of these factors is diluted when cells are disrupted and addition restores more or less normal concentrations.

The work of Cohen and Hayano, Ratner and Pappas (17), Ratner and Petrack (18), Jones, Spector and Lipmann (19) and of Hall and Cohen (20), carried out between 1946 and 1957, added carbamoyl phosphate ($\text{NH}_2 \cdot \text{CO} \cdot \text{O} \cdot \text{PO}_3\text{H}_2$) and arginino-succinate to the series of intermediates of the ornithine cycle. Arginino-succinate is formed from citrulline and aspartate and reacts further to give arginine and fumarate according to the following reactions:



These discoveries led to the present concept of the ornithine cycle:—



This new development not only clarified details of the intermediate stages but also showed that the second nitrogen atom of urea can be derived directly from amino nitrogen, without ammonia being an intermediate. The amino-nitrogen of aspartate is formed by transamination from glutamate, and glutamate can obtain its amino group either by transamination with other amino acids or from ammonia.

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 piece by piece the barriers of ignorance.

Some Reactions of the Scientific Community

The great majority of scientists accepted the evidence presented in 1932 as convincing and commented enthusiastically. Warburg arranged a formal invitation for me by the Kaiser Wilhelm Gesellschaft, signed by Max Planck, the President, to speak on the subject in Berlin. Meyerhof invited me to lecture at Heidelberg. Hopkins (21) referred to it in his Presidential Address at the Royal Society in November 1932. 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 a Russian physiologist of Leningrad, F. S. London, argued that ornithine had no effect in his experiments on the isolated perfused dog liver (22). In 1942 Trowell of the Cambridge Physiological Laboratory reported that the perfused rat liver did not respond to ornithine (23). At the same time Bach and Williamson (24) 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 synthesise urea without participation of arginase. As late as 1956 Bronk and Fisher (25) reported – from my own Department at Oxford – that under certain conditions citrulline is less effective than ornithine in promoting urea formation from ornithine and they concluded that citrulline cannot be an intermediate. The results of London and of Trowell were due to 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. While 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 the postulated kinetic behaviour. 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.

Comparative Biochemistry of the Ornithine Cycle

Already in 1933 it was established that the ornithine cycle occurs generally in ureotelic species, i.e. in the animals which excrete their surplus nitrogen in the form of urea (26). These include mammals, amphibians and the reptiles belonging to the order of Chelonia (turtles, tortoises).

An interesting development was the demonstration by Srb & Horowitz (27) in 1944 that the mould *Neurospora* can synthesise arginine via ornithine through the reactions of the ornithine cycle, with citrulline as an intermediate stage. Later many investigators showed that this is also true generally for micro-organisms and for plants (28).

Thus the detoxication of ammonia in vertebrates utilizes a pathway evolved already at very low levels of life for biosynthetic purposes.

Uric Acid Synthesis in Birds

Retrospectively I must say that I was lucky in selecting the pathway of urea synthesis as the first problem in the study of biosyntheses. It is now obvious that the solution of this problem was much simpler than that of similar cases where progress depended largely on the availability of isotopes as tools, and these did not become generally available until after World War II.

After the successful work on the urea synthesis, I began at once to tackle the problem of uric acid synthesis in birds, a process physiologically analogous to urea synthesis, being the main process in birds by which ammonia is detoxicated and neutralized. This work was undertaken together with another medical student, T. H. Benzinger (29). We used pigeons for the first experiment because it was the most readily available bird for experimental purposes. To our great surprise, the liver of pigeon liver slices failed to form uric acid whilst chicken liver slices when tested gave clear-cut positive results. Since the pigeon is no exception to the rule that birds excrete their excess nitrogen mainly in the form of uric acid, we searched other organs for their capacity to form uric acid, and in the course of these experiments we discovered that when slices of liver and kidney are incubated together, or when the medium in which liver slices had been shaken is subsequently incubated with kidney slices, uric acid is formed. Kidney slices alone did not form much uric acid. These findings raised the question of the product formed by the liver and subsequently converted to uric acid by the kidney.

The advent of Hitler and my dismissal from my position at Freiburg University forced me to discontinue this work. Four years later I had the opportunity of following up these earlier observations in England at Cambridge and at Sheffield Universities together with Dr N. L. Edson (30) of Otago University, New Zealand, and Dr A. Öström (31) of Stockholm. This work led to the discovery that pigeon liver synthesises hypoxanthine from ammonia and unknown carbon precursors and that the long established enzyme xanthine oxidase present in pigeon kidney converts hypoxanthine to uric acid. We found that glutamine and oxaloacetate accelerate the rate of hypoxanthine synthesis (facts which later proved highly significant) but we were unable to establish details of the intermediary stages leading to hypoxanthine.

In the hands of others the biosynthesis of hypoxanthine turned out to be a very complicated process involving more than a dozen intermediary reactions. These were clarified in the course of the 1950's, mainly with the help of isotopic tracers. The purine skeleton is built up on the nitrogen atom of ribosylamine 5-phosphate. Substrates utilized in this process include glutamine, glycine, aspartic acid, carbon dioxide and a one-carbon fragment, equivalent to formaldehyde which is supplied in the form of formyltetrahydrofolic acid. This work led to the realization that the pathway of purine synthesis for the formation of nucleic acids is the same as that leading to uric acid in birds. Pigeon liver which synthesizes the purine ring at great speed therefore proved a most useful material on which to study nucleic acid synthesis.

Thus we meet again the phenomenon that a general metabolic pathway, that of nucleic acid synthesis, which occurs in lower organisms has been utilized in the course of evolution to serve as a detoxication mechanism for ammonia in higher organisms.

Clinical Aspects

The initial work on the ornithine cycle was carried out when I was working as a clinician with full responsibility for patients in the Department of Internal Medicine at Freiburg University. I was convinced at that time that studying intermediary metabolism in an academically orientated hospital was justified because I felt that sooner or later any information on intermediary metabolism would be relevant to clinical problems. In the long run my expectations were not disappointed. Diseases are now known where intermediates

of the urea cycle accumulate in the body fluids and the urine. Inborn errors affecting four of the enzymes of the ornithine cycle have been reported. When ornithine transcarbamylase is defective, ammonia accumulates and gives rise to hyperammonaemia. Citrulline accumulates when the enzyme responsible for its removal (argininosuccinate synthetase) is lacking and argininosuccinate accumulates when the lyase responsible for its breakdown is defective. Hyperargininaemia occurs when the activity of arginase is low. These conditions have been observed in young children and in all types mental retardation is the main clinical sign.

The diagnosis of these conditions is not merely a matter of academic interest. I have recently been associated with the treatment of a case of ornithine transcarbamylase deficiency where the biopsy of the liver showed that about 2% of the normal activity of the enzyme was still present. As a result of careful dieting which makes maximum use of the residual enzyme activity, the child is now making good progress. The protein intake has to be restricted and it has to be distributed as evenly as possible throughout the day so that at no time is the liver overloaded with ammonia.

General Comments

The story of the ornithine cycle illustrates the importance to progress of new techniques, especially of techniques which make it possible to conduct a large number of experiments, and of studying a phenomenon under many different conditions, with a view to establishing factors which affect the rate of the process. It also illustrates the importance of following up an unexpected and puzzling observation arising in the course of the experiments. Luck, it is true, is necessary but the more experiments are carried out the greater is the probability of meeting with luck. The story also shows that adverse criticisms are liable to be raised on the grounds that either the observations are not confirmed or that some other observations do not fit in with the interpretation of the findings. Almost every major development in science meets with criticisms of this kind.

(This article is based on a lecture given at a symposium on Ammonia Metabolism at Budapest in May 1972 (see *Ammoniakstoffwechsel (Ammonia Metabolism)*, edited by I. Szám, F.K. Schattauer Verlag Stuttgart, New York 1972, page 7.)

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Book Review – Perfusion Techniques in Biochemistry

A Laboratory Manual in the use of Isolated Perfused Organs in Biochemical Experimentation. By B.D. Ross. Clarendon Press: Oxford University Press, £11.50 net (1972).

The perfusion of isolated organs for physiological and biochemical studies has been practised for over a hundred years but recently, especially during the last ten years, this technique has gained greatly in importance. One reason is the increasing importance of experimentation on whole organs of higher animals, as opposed to tissue fragments and isolated enzymes. More and more biochemical research is now concerned with the integrated function of organs and the correlation between biochemical activities and physiological function. It is one of the major aims of current biochemical work to elucidate the mechanisms which regulate the rates of metabolic processes. Many aspects of these problems must be investigated in the intact organ where the enzymes are located in their natural setting and where the normal interplay between the enzymes is undisturbed. Of course complementary work of isolated pure enzymes is no less important.

A second reason for the increased use of organ perfusion is the fact that it has become possible to perfuse the organs of small laboratory animals such as the rat. Liver, kidney, heart muscle, skeletal muscle, intestine, endocrine glands, brain, lung and many other organs have recently been studied in this way. This development was opened by improved surgical techniques and by the progress in micro-analytical techniques. Reliable quantitative data can now be obtained on fractions of a millilitre.

The book describes the present state of the techniques available. It is the only modern review of its kind. Dr. Ross has had many years of practical experience in the field of organ perfusion and was therefore well qualified for the task he set himself. Technical information, together with critical comment, is offered in much more detail than can usually be given in a journal. In all 71 methods of perfusion are described for 22 organs and tissues. As a laboratory manual the book, bringing together relevant information from many sources in a handy form, should prove an invaluable help in every laboratory where organ perfusion is used.

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