Liversidge Research Lecture

No. 10
1954

CHEMICAL STRUCTURE AND BIOLOGICAL FUNCTION OF THE PYRROLE PIGMENTS AND ENZYMES

M.R. LEMBERG

The Royal Society of New South Wales

Max Rudolph Lemberg

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MAX RUDOLPH LEMBERG 1896–1975

Max Rudolph (Rudi) **Lemberg** was born in Breslau, Germany, on 19 October 1896 into a Jewish family. He was educated at a humanist gymnasium in Breslau, then studied chemistry, physics, mineralogy and geology at Breslau University, and subsequently at the universities in Munich and Heidelberg, until 1917. His studies were interrupted by enlistment in the German army in the First World War in which he was awarded the Iron Cross (2nd class) for his daring in attempting to repair a telephone line during the battle of the Somme.

In 1919 he resumed his studies at Breslau University where he worked for his Ph.D. with Heinrich Biltz on methyl-substituted uric acid derivatives, and in 1923 he went to Mannheim to work with Bayer. In 1926 an economic crisis resulted in his retrenchment from Bayer, whereupon he went to Heidelberg to work with Freudenberg, funded by a grant from the Notgemeinschaft der Deutschen Wissenschaft. He worked on catechins for six months, but then independently undertook studies of the chromoproteins in red algae. He was awarded his Habitation as Privatdozent (lecturer) at the University of Heidelberg in 1930, and with Freudenberg's recommendation Lemberg was awarded a Rockefeller Foundation Fellowship to work under Gowland Hopkins at the Sir William Dunn Institute of Biochemistry at Cambridge University. He returned to the University of Heidelberg in 1931, and soon afterwards was appointed as Privatdozent and Assistant in the Inorganic Chemistry Department, but the rise of antisemitic Nazism caused Lemberg and his wife to flee to England in 1933. He went back to Cambridge to work with Hopkins, with help from the Academic Assistance Council (later to become the Society for Protection of Science and Learning), a body started by Rutherford and Hopkins to assist scientists fleeing the Hitler terror.

In 1935, with support from Hopkins, Lemberg was appointed as a director of the Research Biochemical Laboratories in the Institute of Medical Research at Royal North Shore Hospital, Sydney. His growing reputation in the chemistry of biological pigments earned him the invitation to write the first review on this topic for the Annual Review of Biochemistry of 1937. Over the next 40 years he worked on many aspects of the chemistry of naturally occurring porphyrins (haematins, urobilins, urobilinogens, bilichrysins) – the blood and bile pigments. During the Second World War (1939-1945) Lemberg's research was diversified to assist in the war effort, but after that, he re-focussed on porphyrin chemistry and soon gained world-wide recognition in this field with his monograph, written jointly with J.W. Legge, "Hematin Compounds and Bile Pigments", Interscience Publishers (New York and London, 1949).

In 1955 Rudi Lemberg founded the Australian Biochemical Society and became its first president. In 1957 he was Vice-President of the Australian Academy of Science, and he was Visiting Professor, University of Pennsylvania, Philadelphia in 1966. His impressive contributions to the chemistry and biochemistry of porphyrins may be summarised as follows:

1. structural studies of bile pigments
2. the mechanism of conversion of hames into bile pigments
3. the structure of the prosthetic groups of cytochrome and a cytochrome oxidase, and
4. the biochemistry of metabolic disorders manifested by excess of porphyrins in blood and urine (porphyrines).

In his later years Lemberg co-authored a second major monograph – this time, with J. Barrett, entitled "The Cytochromes", Academic Press (London, 1973).

Honours and Awards.

1948 H.G. Smith Medal, R.A.C.I.

1952 FRS

1953 Coronation Medal

1954 FAA (a Foundation Fellow of the Australian Academy of Science)

1954 Liversidge Research Lecture, Royal Society of New South Wales

1956 Professor Emeritus, Heidelberg University

1956 Foreign Member, Heidelberg Academy of Science

1958 Member, Acad. Anatomico-Chirugica di Perugia

1965 James Cook Medal, Royal Society of New South Wales

1966 Britannica Australia Award for Science

1970 D.Sc., *honoris causa*, University of Sydney

1971 Walter Burfitt Prize and Medal, Royal Society of New South Wales

Biographical Sources

1) Barrett, J. and Robertson, R.N., "Max Rudolph Lemberg", *Historical Records of Australian Science*, 1979, **4**, 133-156.

2) Rimington, C. and Gray, C.H.., "Max Rudolph Lemberg, 19 October 1896 – 10 April 1975: elected F.R.S. 1952", *Biographical Memoirs of Fellows of the Royal Society*, 1976, **22**, 257-294.

Scientific Publications of M.R. Lemberg

Lemberg was the author/co-author of 149 scientific publications that are listed in appendices to the biographical articles (1) and (2) given under Biographical Sources.

Liversidge Research Lecture No. 10, 1954

CHEMICAL STRUCTURE AND BIOLOGICAL FUNCTION OF THE PYRROLE PIGMENTS AND ENZYMES*

M.R. LEMBERG
Institute of Medical Research, Royal North Shore Hospital, Sydney, N.S.W.

Mr. President, Members of the Royal Society, Ladies and Gentlemen,

It is a testimony to the far-sightedness of Liversidge, to whom we owe these lectures, that the aim of the bequest is today as essential and its terms as well-conceived, as they were at the time of his life and death. It is, indeed, difficult for me to imagine that sixty-three years separate the short time which Liversidge spent with Michael Foster at the Cambridge Physiology School from the time of my collaboration with Sir Joseph Barcroft at the same school, and with Sir Frederic Gowland Hopkins at its daughter school of Biochemistry; the same number of years separate our arrivals in Australia.

It was during the years Liversidge was still at Cambridge, in 1871, that Hoppe-Seyler laid the foundations of the field of knowledge which I am going to discuss, by his conversion of haemoglobin to a porphyrin, followed a few years later by his discovery that the same type of compound could be obtained from chlorophyll.

When the Royal Society entrusted me with this lecture, I felt somewhat diffident, knowing that I had chosen a general survey of the pyrrole field as my subject for the Presidential Address of Section, N of A.N.Z.A.A.S. at the Canberra January meeting, and that I should be in danger of repeating what I had said there. Permit me, therefore, to put before you the story of some of my own adventures in this field. If such a procedure is perhaps ill-suited for a ceremonial lecture such as this - there is a good excuse to be found in Liversidge's terms, i.e. that these lectures should be designed to encourage research. Nothing is more needed for the encouragement of research than the demonstration that a research career is a great intellectual adventure compared with which "mere physical adventure is a pale and colourless experience". This phrase is taken from a lecture of Frederic Wood-Jones, F.R.S., entitled "The Spirit of Adventure", and found in his book "Life and Living", a lecture which every student, research worker and university teacher should read. As in all adventures, hardships and uncertainties are part of the game and heighten its enjoyment.

It is the story of a long research adventure which I intend to put before you. Everyone of us, however, explores only a few corners of a continent, and a rough map of what is known today must precede the story. The field is that of the tetrapyrrole pigments and enzymes, full of intrinsic chemical interest, but still more fundamentally important for the physiologist and biologist. Structure, metabolism, function and their correlation will therefore receive attention.

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*Liversidge Research Lecture delivered to the Royal Society of New South Wales, July 15, 1954. Reproduced by permission of the Royal Society of New South Wales from *J. Proc. Roy. Soc. N.S.W.*, 1954, **88**, 114-135.

Biological Importance

There are three fundamental biological processes - two of them closely linked up - for which tetrapyrroles are essential (Fig. 1).

Firstly *photosynthesis*. The elucidation of this process by which sunlight energy is converted into the potential energy of foodstuffs may one day bring humanity more benefits than atomic energy, and certainly less danger and misery. Several chlorophylls, e.g. *a* and *b* in higher plants, or bacterio-chlorophyll in purple bacteria, and the bile pigment-chromoproteins of red and blue algae, phycoerythrin and phycocyanin, are involved in photosynthesis. In addition, we know that iron-porphyrin (haem) complexes bound to protein are also required, such as cytochrome *f* in the chloroplasts of green plants, cytochrome c_2 in purple bacteria.

Photosynthesis	Chlorophylls
	Phycochromoproteins
	Haem enzymes
Cellular respiration	Cytochrome oxidase
	Cytochromes
	Peroxidases
	Catalases
Oxygen storage and transport	Myoglobins and haemoglobins
	Chlorocruorin

FIGURE 1. Biological role of tetrapyrroles

Secondly, *cellular respiration*. We may define life as a complex organisation by which a steady state of a free energy content far above that of the equilibrium is maintained by a constant influx of energy needed for the maintenance of the steady state and of the complex organisation itself. This energy is provided in most cells by the stepwise oxidation of food materials, and in the chain of events which require the movement of electrons from the substrates to atmospheric oxygen, haemo- proteins such as cytochrome oxidase and several cytochromes are involved. The peroxidases and catalases serve as auxiliaries in this process. An overwhelming part of life on earth depends on the systole and diastole of these two processes photosynthesis and cellular respiration. Only a few autotrophic and a few strictly anaerobic bacteria form an exception; some of the autotrophs at least, e.g. the nitrate reducing bacteria, probably also use cytochromes, and some obligatory anaerobes contain a little catalase.

Thirdly, when an organism grows in bulk and complexity, diffusion is no longer sufficient to bring the oxygen for cellular respiration from outside. Special *oxygen carriers* in the blood are required. Haemoglobins are found in moulds, e.g. in yeasts, and in leguminous root nodules, but their essential function appears to begin sporadically in Invertebrates, to become regular in Vertebrates, with the recently discovered exception of a few fishes. Finally, the red muscle which is able to store oxygen combined with myoglobin, is a far more efficient organ than the white muscle which lacks it.

Chemical Structure

If we now turn to chemical structure, we find that the structural basis of all these compounds is the porphin ring (Fig. 2), directly for haem compounds and chlorophylls, more indirectly for the bile pigments.

Porphyrins are porphin substituted with various side chains at the eight β-positions of the four pyrrole rings; haems are their internal tetracoordinate iron complexes, chlorophylls magnesium complexes, usually of dihydro- or tetrahydroporphyrins. Finally, bile pigments are essentially porphyrins whose ring has been opened by oxidative scission.

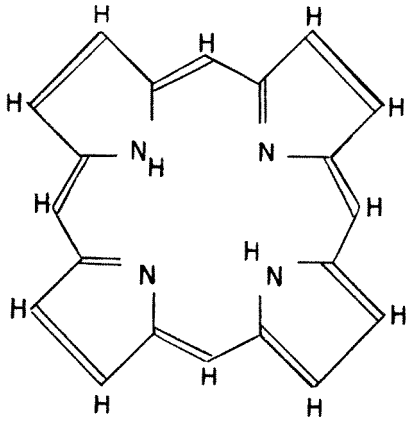


FIGURE 2. Porphyrin

TABLE 1
Porphyrins and Their Occurrence as Metal Complexes and Prosthetic Groups

Porphyrin	Side Chains	Occurrence as Prosthetic Group or Metal Complex
Aetio-III	4M, 4E	O
Meso-IX	4M, 2E, 2P	O
Proto-IX	4M, 2V, 2P	Fe: haemoglobins, myoglobins cytochromes *b* catalases, horse radish peroxidase (Mg: Chlorella mutant)
Haemato-IX	4M, 2HE, 2P	Fe: (modified) cytochrome *c*
Acetyldeutero-	4M, 2P, lAc	Fe : lactoperoxidase
Chlorocruoro-	4M, IV, IF, 2P	Fe: chlorocruorin
Cyto-(*a*)-	1F, I long alkyl	Fe cytochrome oxidase, cytochrome *a*, a_1
Copro-I and III	4M, 4P	(Cu, Zn: urine)
Uro-I and III	4AC, 4P	(Cu: turacin) (Zn: urine)

Abbreviations:

M = methyl, $-CH_3$ — F = formyl, -CHO

E = ethyl, $-C_2H_5$ — AC = acetic acid radical, $-CH_2CO_2H$

V = vinyl, $-CH=CH_2$ — P = propionic acid radical, $-CH_2CH_2CO_2H$

HE = hydroxylethyl, $-CHOHCH_3$

Of the porphyrins (Table 1), protoporphyrin, with four methyl, two vinyl and two propionic acid side chains, is the most important. It forms the prosthetic groups of haemoglobins and myoglobins, of a number of cytochromes, such as *b* and *f* (and in somewhat modified form of cytochromes *c*), of catalases and of some peroxidases.

Porphyrins with a formyl side chain are found as the prosthetic groups of cytochrome oxidase (the Atmungsferment), of cytochrome *a* and of the oxygen carrier in the blood of Sabellid worms, chlorocruorin.

Acetyl groups are found in bacteriochlorophyll and in the peroxidase in milk (Morell, 1953).

Coproporphyrins with four, and uroporphyrins with eight carboxyl groups in their side chains, are found, usually in small amounts as free porphyrins, occasionally as copper or zinc complexes. They will mainly interest us in connection with the biosynthesis of haem.

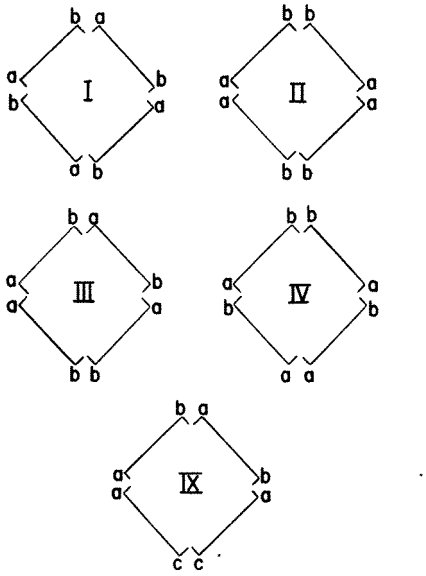


FIGURE 3. Porphyrin isomers

Porphyrins with two types of side chains (e.g. copro- or uroporphyrin) can form four isomerides (Fig. 3), but only two, types III and I, have so far been found in nature. All functionally important compounds, chlorophylls as well as haem compounds, are derived from type III. Porphyrins with three types of side chains, such as protoporphyrin, can form 15 isomers; the natural protoporphyrin IX is derived from type III.

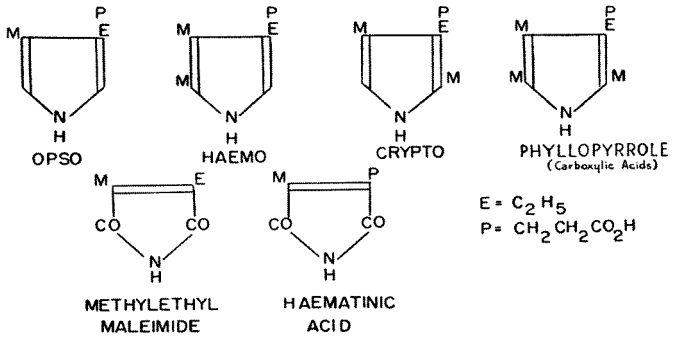


FIGURE 4. Products of reductive and oxidative scission

The correct formula for the porphin ring was given by Wilhelm Küster (Küster and Deihle, 1913) based on his studies on the products of oxidative scission, substituted maleimides, and on the studies of other workers on the products of reductive scission with hydroiodic acid, substituted pyrroles (Fig. 4). This evidence culminated in the synthesis of protoporphyrin and haemin by H. Fischer in 1929 (cf. Fischer, 1937, p. 372). Monopyrroles are first condensed to two dipyrrolic pyrromethenes and then two of these to the unsymmetrically substituted type III porphyrin.

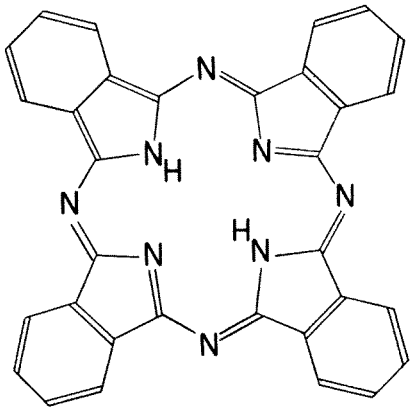


FIGURE 5.- Phthalocyanine.

Finally the structure has been confirmed by purely physical methods. Phthalocyanine (Fig. 5), a tetrabenzenotetrazaporphin, synthesised by Linstead (1934) was the first organic compound to give a complete Patterson X-ray diagram in the hands of Robertson (1935). The molecule is flat and there is no real difference between the four isoindole rings, although the symmetry only approaches the tetragonal one. Linstead has bridged the structural gap between phthalocyanin and porphin by synthesising tetrabenzo- and tetraazoporphyrins. The central 16-membered ring (Fig. 7) leaves a hole in the centre with a diameter of 2.65 Å, just large enough to be filled by atoms such as iron (atomic diameter, 2.54 Å.), but the ring is also adjustable by small alterations of the length of, and angle between, its many bonds, to take larger atoms such as Mg, and to bind even atoms like Be, otherwise never found in planar tetracoordination.

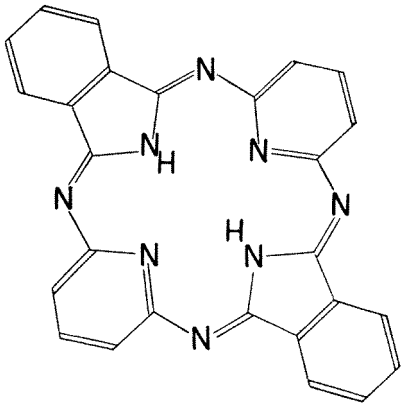


FIGURE 6. Dipyridine-diisoindole macrocyclic ring system of Linstead.

Recently Linstead (1953) has synthesised similar macrocycles with two of the four isoindoles replaced by either pyridine or benzene. The dibenzenediisoindole compound no longer forms metal complexes; it lacks the possibility of tetracoordination. The dipyridine-diisoindole compound (Fig. 6) forms such complexes. It is of interest that the

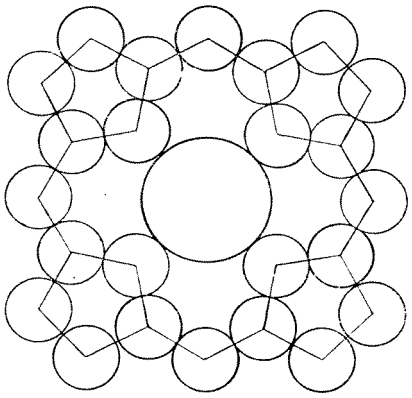


FIGURE 7. Stereochemistry of porphin. The central circle represents the central hole filled by two hydrogen atoms in porphyrins and by the metal atom in the metal complexes. The four adjoining circles are those of the four pyrrole nitrogen atoms, the remaining circles represent carbon atoms.

free compound has a spectrum quite different from that of porphins, whereas those of the metal complexes resemble porphin complexes. In the free compound the hydrogen atoms are evidently strictly bound to the two isoindoles, thus severely restricting resonance which is still possible in the metal complex.

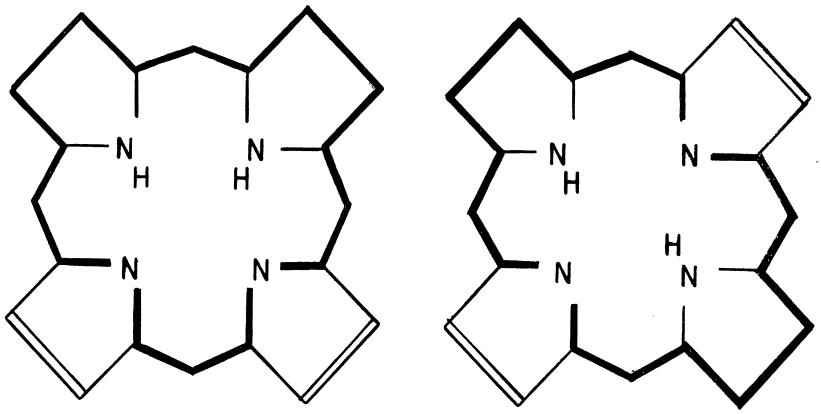
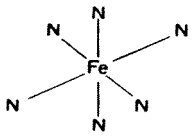


FIGURE 8. Porphyrin tautomerism

The type of linkage of the central hydrogens in the free porphyrins is not yet fully established. It appears that free porphyrins consist of mixtures of two tautomeric forms (Fig. 8), each in turn stabilised by resonance of several canonical forms.

Iron in the *haem compounds* can be bound in two different ways. In addition to the four valencies going to the four nitrogens of the porphin ring, there are two additional sites of coordination above and below the porphin plane (Fig. 9). Ferrous haem binds, e.g. two



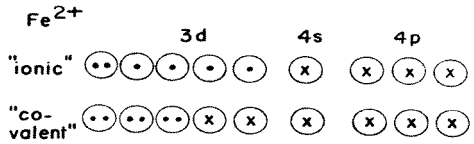


FIGURE 9. Iron porphyrin (haem) compounds

TABLE 2
Chlorophylls and Related Compounds.

	State of Ring	Side Chains	Isocyclic Ring C_6-C_γ	X in $-CH_2CH_2CO_2X$
Chlorophyll *a*	Mg dihydro-porphin	4M; IE, IV, IP	$-CO-CH(CO_2M)-$	phytol
Protochlorophyll	Mg porphin	"	"	phytol
Vinylphaeoporphyrin a_5	Mg "	"	"	H
Chlorophyll *b*	Mg dihydro-porphin.	3M, IF; IE, IV, 1P	$-CO-CH(CO_2M)-$	phytol
Bacteriochlorophyll	Mg tetra - hydroporphin	4M; IE, IAc. IP	$-CO-CH(CO_2M)-$	phytol

Phycochromoproteins.

Phycocyanin	biladiene- (*a,b*)	4M, 2E, 2P	protein, no metal
Phycoerythrin	" + 1H	"	"

molecules of pyridine to a hexacoordinate complex, called haemochromogen, or more concisely haemochrome. In this instance the linkage, called "covalent", is of d_2sp_3 type, using the 3d, 4s and 4p orbitals of iron, and the molecule is diamagnetic. In other compounds, however, the paramagnetism of ionic iron is preserved. The linkages, not quite fortunately called "ionic", are also covalent, but involve only the 4s, 4p and perhaps 4d orbitals of iron.

Chlorophyll (Table 2) contains Mg instead of the iron of haem. Figure 10 shows the relationship between haem and chlorophyll *a*. We may imagine that one of the propionic acid groups of haem is curled up and oxidatively condensed to a fifth isocyclic ring. This oxidation is partly counterbalanced by hydrogenation of the nucleus (transforming porphin to dihydroporphin or chlorin) and reduction of one vinyl to ethyl. Finally the carboxyl groups are esterified, one with methyl thus protecting the β-keto acid carboxyl, and one

with the long chain aliphatic alcohol phytol. We shall see below that this picture is not merely imaginary.

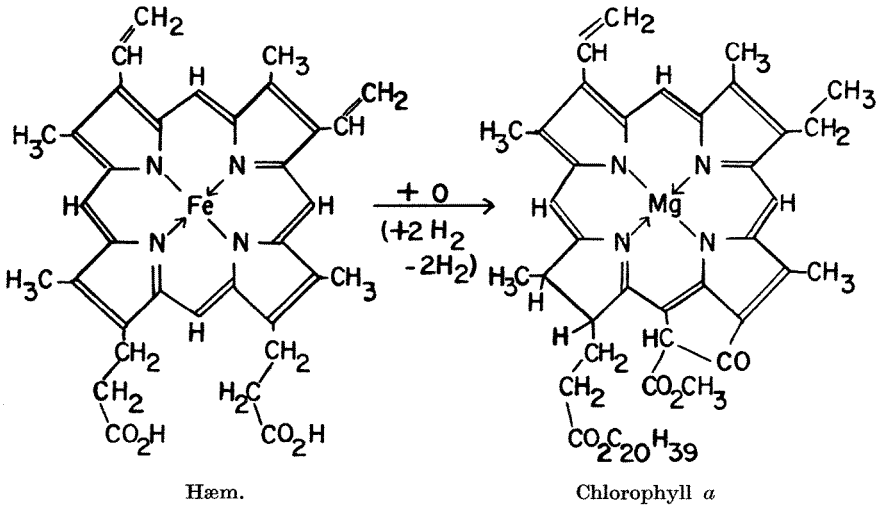


FIGURE 10. Relationship between haem and chlorophyll *a*

Protochlorophyll from which chlorophyll *a* arises on irradiation in the plant is still a porphin, not a chlorin derivative. Chlorophyll *b* carries a formyl instead of a methyl side chain of chlorophyll *a*. Bacteriochlorophyll is a tetrahydroporphin with an acetyl side chain.

In contrast to the porphin derivatives, the *bile pigments* are compounds with an open tetrapyrrolic chain, although in many instances they are probably more correctly formulated as rings closed by a hydrogen bond; this holds at least for their metal complexes. In the porphin series there is only one stable hydrogenation state of the central ring system, which remains essentially intact if hydrogen is introduced into one or two crossed double bonds to form dihydro- and tetrahydroporphins. Porphyrinogens with four $-CH_2-$ bridges are rather unstable substances, probably for stereochemical reasons. No compounds are known which contain $-CH_2-$ and $-CH=$ bridges. This is different in the bile pigments (Fig. 11) where several classes exist, varying in colour from white to yellow, red, violet and blue-green, which differ in number of conjugated double bonds. Wherever a $-CH_2-$ group replaces a $-CH=$ bridge, the conjugation becomes interrupted. The fully conjugated bilatrienes or verdins correspond to the porphyrins, the leuco-compounds or bilanes to the porphyrinogens. But between them we find systems with only two (urobilins), twice two (rubins), or three pyrrole rings (violins), belonging to conjugated systems. It is this field to the development of which my first studies contributed.

Chromoproteins of Red and Blue Algae. My starting point was a sentence in Kostytschev's well-known text of plant physiology. Speaking of the chromoproteins of red and blue algae, he concluded: "Their chemistry has not been sufficiently investigated." These beautifully crystalline, strongly fluorescent proteins, phycoerythrin and phycocyanin had attracted the attention of many botanists (Engelmann, Gaidukov, Molisch, Boresch, Kylin). It had been shown that they acted as photosensitisers of the photosynthesis of red algae, allowing them to penetrate into deeper layers of the sea than green or brown seaweeds. The absorption of phycoerythrin and phycocyanin is maximal in the orange to green part of the spectrum, where that of chlorophyll is small. This is of particular importance in deeper layers, where red light no longer penetrates. Some Cyanophyceae also show the interesting phenomenon of "complementary chromatic adaptation".

Irradiated with coloured light, they change their colour to one roughly complementary to that of the incident light.

The interest of the botanists in these compounds has recently been revived by the findings of Haxo and Blinks (1950) that light energy absorbed by phycoerythrin or phycocyanin is used more efficiently for photosynthesis of some algae than is light

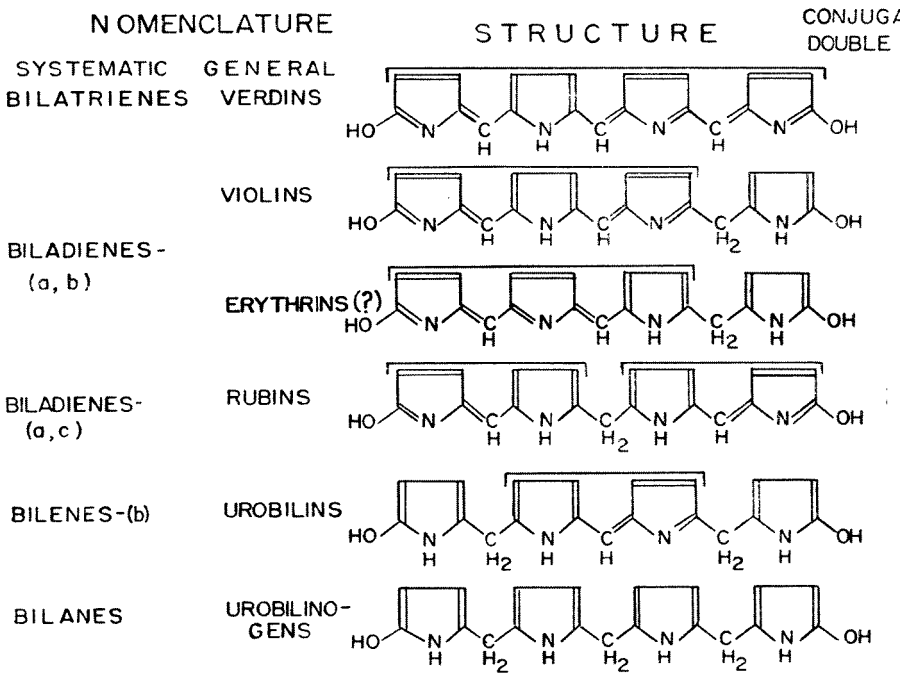


FIGURE 11. Bile pigments
The brackets over the pyrrole rings indicate those which form a system of conjugated double bonds.

absorbed by the chlorophylls. While it appears that chlorophyll *a* is necessary for the energy transfer in these algae, the explanation of the phenomenon is not yet clear (cf. French and Young, 1952; Duysens, 1951).

Kitasato (1925) had failed to find any evidence for the pyrrolic nature of the prosthetic group, but I suspected that his experiments were inconclusive. I used the very suitable starting material which Kitasato had described, a Japanese delicacy called "nori" prepared from *Porphyra*, a red alga. At the outset I struck the great difficulty that the prosthetic groups of both the red phycoerythrin and the blue phycocyanin was far more firmly bound to the protein than in haemoglobin, and new methods had to be devised to obtain them free from attached peptide. Once this was done, it was easy to demonstrate the pyrrolic nature, and the strong fluorescence of the zinc complexes placed them into close relationship to urobilins (bilenes). The prosthetic groups of phycoerythrin and phycocyanin were identified with two new compounds, mesobiliviolin and mesobilierythrin (see Fig. 11) which could be obtained by ferric chloride oxidation of mesobilirubinogen (mesobilane), the leuco compound of bilirubin (Lemberg, 1930). Thus two new types of bile pigments were obtained which were later converted into the crystalline mesobilirubin and mesobiliverdin by alcoholic potash (Lemberg and Bader, 1933).

There is still much work to be done in this field for a chemist who is not afraid to start with a few hundred litres of extract to end up with a few milligrammes of substance. The structure of the prosthetic group of phycoerythrin and its relationship to that of

phycocyanin is not yet safely established, and this is of particular interest for the problem of complementary chromatic adaptation, which is based on the relative increase of the substance, phycoerythrin or phycocyanin, absorbing most of the light.

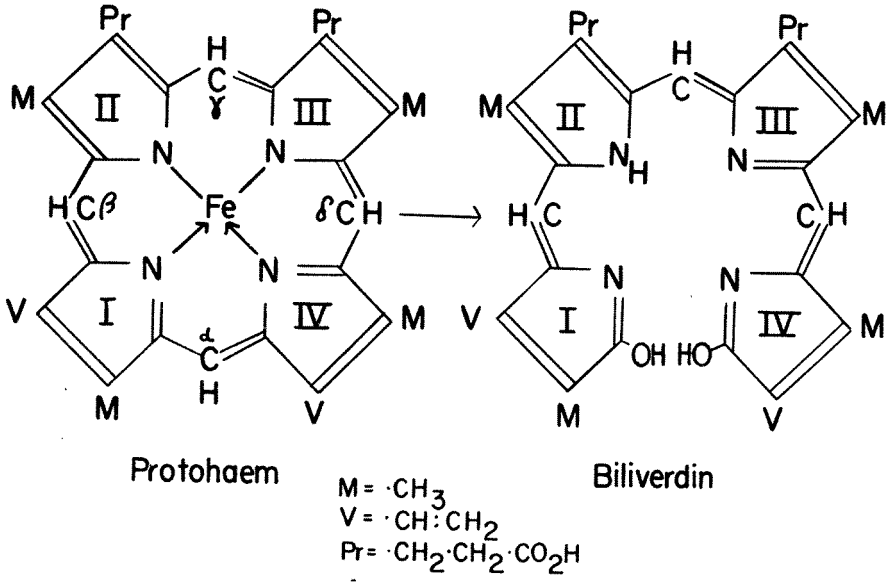


FIGURE 12. Relation of biliverdin to protohaem

The type of linkage between prosthetic group and protein required further study. Apart from the firm peptide linkage, there is a second weak linkage easily broken by dilute acid. It is this linkage which is necessary for the strong fluorescence of the native chromoproteins and for their extraordinarily strong absorption of light.

The biogenesis of these and other invertebrate bile pigments, such as that found in the haemolymph of insects (Hackman, 1952), e.g. our common cicada, appears to be different from that in Vertebrates, and the nature of the side chains indicates neither formation by oxidation from haem compounds, as in the Vertebrates, nor photooxidation of chlorophyll.

Bile Pigments. The next step was an attack on another class of bile pigments. Oocyan, the blue-green pigment of many birds' egg shells, e.g. of the duck and the emu, was the first pigment of the bilatriene class to be isolated in pure form (Lemberg, 1931). It is this compound which causes the blue-green colour in the well-known Gmelin or Fouchet reaction for bile pigment. At that time I worked in Hopkins' laboratory as a Fellow of the Rockefeller Foundation. There was an atmosphere of adventure in which the Institutes of Hopkins, Barcroft and Keilin were closely linked. One of those days Keilin brought Barcroft to me to discuss a green pigment in the dog's placenta which disturbed Barcroft's attempts at haemoglobin estimation. In a short time I had identified this substance, uteroverdin, with oocyan and established its structure as that of dehydrobilirubin or bilatriene (see Fig. 11) (Lemberg and Barcroft, 1932). Later it was prepared from bilirubin by dehydrogenation (Lemberg, 1932). Uteroverdin had been intensively studied by early biologists and embryologists of the German and French schools. It was the careful study of this old literature in the peaceful atmosphere of the Old Cambridge Library which made me first doubt the primogeniture of bilirubin. Its correct structure showed biliverdin to be more closely related to haemin than is bilirubin (Fig. 12). In one of my first studies in Australia I described the reduction of biliverdin to bilirubin in liver slices, and the enzyme systems which can use biliverdin as hydrogen acceptor (Lemberg and Wyndham, 1936). This reduction (Fig. 13) is continued in the

mammalian organism by bacterial enzymes in the intestine ending in a mixture of mesobilane and tetrahydromesobilane, known as urobilinogen or stercobilinogen; in the literature you find these names urobilinogen and stercobilinogen confusingly applied to mesobilane and tetrahydromesobilane respectively (cf. Lemberg and Legge, 1949, p. 134). The structure of tetrahydromesobilane suggested by Fischer is open to doubt and requires reinvestigation.

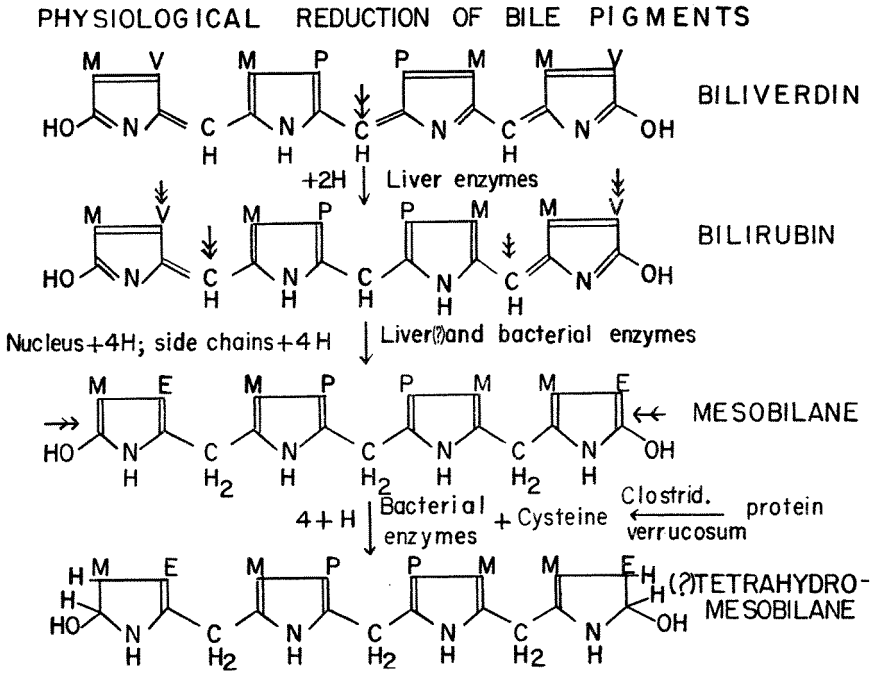


FIGURE 13. Physiological reductions of bile pigments.
The double arrows indicate the part of the molecule at which reduction takes place.

Bile Pigment Formation. The knowledge of the properties of biliverdin led directly to the next step, the explanation of the transformation of the haem compounds to bile pigments. It was one of the rare instances in which a reinterpretation of data of other workers allowed one to predict the outcome of a study with some degree of certainty. At that time, Warburg (1932) classified the haemins into three classes according to their colour, red haemins derived from protoporphyrin, green haemins derived from chlorophylls, and dichroic green-red haemins such as the prosthetic group of the Atmungsferment, derived from what we now know to be formylporphyrins. Warburg and Negelein (1930) had formed such a "green haemin" by coupled oxidation of haemin in pyridine solution with hydrazine. Its supposed methyl ester, obtained by the action of methanol hydrochloric acid, had been obtained crystalline. This ester contained four chlorine atoms. Now haemins contain only one, there was little chance of chlorination under the experimental conditions, and I knew that biliverdin readily forms a ferrichloride $[B]^{+}[FeCl_4]^{-}$. I could show that the "green haemin ester" was, in fact, biliverdin ester ferrichloride, and the transformation of haemin to biliverdin had thus been carried out inadvertently (Lemberg, 1935).

The green haemin in itself, however, was still an internal iron complex, but, with an increased lability of its iron; in fact a pyridine haemochrome, which I called verdohaemochrome. It had been formed by the removal of one carbon atom from the ring and by its replacement by oxygen. With ammonia, the oxygen could in turn be replaced by nitrogen, yielding a monoazahaemin with restored firm iron linkage (Lemberg, 1943).

Oxidation of the ring thus precedes iron removal, and no porphyrin is formed as intermediate of haemoglobin breakdown. It took more than ten years of intensive work before this new concept (Fig. 14) became generally accepted.

The first model was still far removed from physiological conditions, but step by step it was brought nearer to them. Hydrazine was replaced by ascorbic acid, haemin in pyridine by haemoglobin at physiological pH and 37° C. Intermediates were observed and a clearer picture of the reaction mechanism obtained (cf. Lemberg and Legge, 1949, Chapter X). The work was not lacking unexpected surprises. Thus haemoglobin gave finally biliverdin, but as intermediate choleglobin with a haem different from verdohaem. The greening of haemoglobin by certain streptococci is due to choleglobin formation, and a cholehaem prosthetic group was later found in the peroxidase of leucocytes (Foulkes, Lemberg and Purdom, 1951).

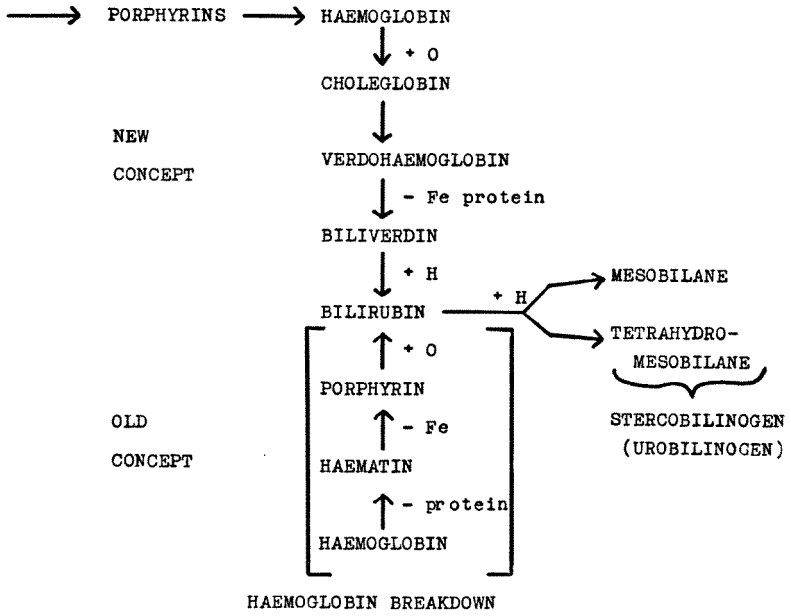


FIGURE 14. Haemoglobin breakdown.

Figure 15 shows the picture as it finally emerged. The ring is at first oxidised but remains still carbon-closed. (The structure of cholehaem is here depicted in the same way as Linstead formulates his first oxidation products of phthalocyanine, but hydroperoxidic structures are not excluded). Later the carbon is replaced by oxygen and Sjöstrand has recently demonstrated that it emerges as carbon monoxide.

INTERMEDIATES IN BILE PIGMENT FORMATION

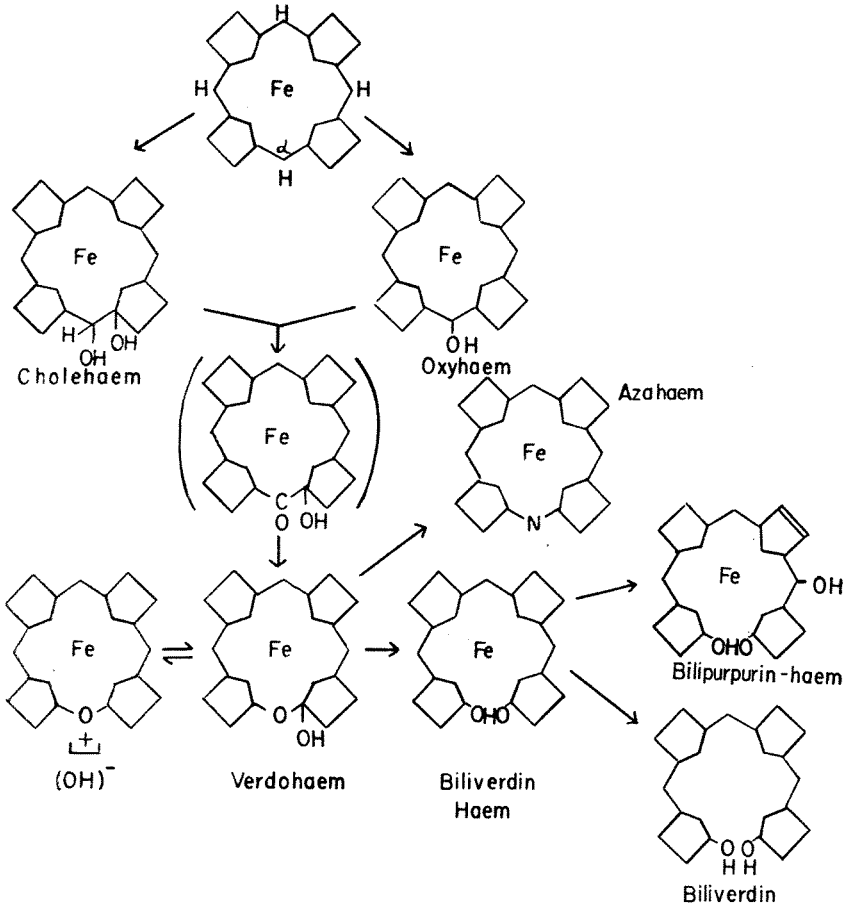


FIGURE 15. Intermediates in bile pigment formation.

This poison is thus not quite unphysiological. Other haem compounds, myoglobin, catalase, methaemalbumin and even free haematin are similarly oxidised. The mechanism is essentially a peroxidative autoxidation of the ring catalysed by the haem iron, in which a $[Fe^{2+}.H_2O_2]$ or $[Fe^{4+}]$ or $[FeO^{2+}]$ complex is involved (Fig. 16).*

Catalase and Peroxidase. This brings the problem in close proximity to the mechanism of the enzymes, catalase and peroxidase. Liver catalase contains a verdohaem group which yields biliverdin by the action of acids (Lemberg, Norrie and Legge, 1939). During its action on hydrogen peroxide, some of the enzyme is destroyed by oxidation of protohaem to verdohaem, but the rate of destruction is greatly increased by ascorbic acid. The inhibition of catalase by ascorbic acid or azide is of a type quite different from that produced by cyanide (Lemberg and Foulkes, 1948).

Foulkes and Lemberg (1948) had obtained apparent spectroscopic evidence for a compound between catalase and ascorbic acid, overlooking that our ascorbic acid solutions contained traces of hydrogen peroxide formed by autoxidation. Chance (1948) found that ascorbic acid accelerated the conversion of a primary complex of catalase with hydrogen

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* The fact that ferric haematin is not reduced to ferrous by ascorbic acid, but can be transformed into bile pigment by ascorbic acid plus hydrogen peroxide (Kench, 1954) is no evidence against this. Ascorbic acid is required for the process and may well reduce an initial $[Fe^{3+}.H_2O_2]$ or $[Fe^{5+}]$ complex to $[Fe^{4+}]$.
$[Fe]$ = haem.

peroxide into a secondary one, which was our supposed ascorbic acid compound and which he formulated as "catalase-H_2O_2 complex II". Such a complex played a normal role in the activity of peroxidases. Finally George (1952, 1953) has shown that the "complex II" of

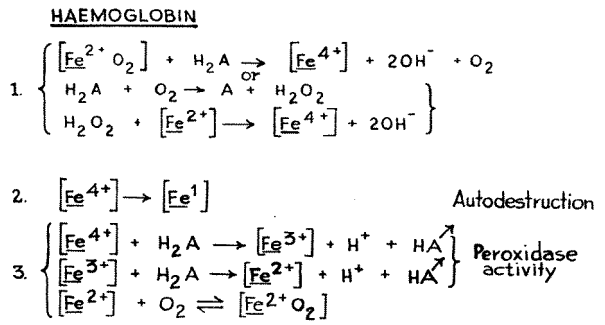

FIGURE 16. Coupled oxidation of haemoglobin and ascorbic acid [*Fe*] haem-iron. H_2A ascorbic acid.

1. Formation of [*Fe* $^{4+}$] directly (first line), or from action of hydrogen peroxide formed by autoxidation of ascorbic acid.
2. Oxidation of porphin nucleus to form choleglobin.
3. Back reduction of [*Fe* $^{4+}$] to [*Fe* $^{2+}$]

peroxidases and of catalase can be obtained by the action of a great variety of oxidants and cannot be formulated as hydrogen peroxide complex. It is best formulated as [*Fe* $^{4+}$] or [*Fe*O^{2+}] complex, and ascorbic acid, or even impurities, cause a monovalent reduction of the primary complex having "effectively pentavalent" iron. It is still too early to say what is the real valency of iron in these compounds, and whether radicals in the porphin ring or protein are formed. One therefore speaks of "effective valency ". Neglecting this we may thus write the action of catalase as in Figure 17. Formulae 1-2 represent the normal catalatic activity of catalase, formulae 3-5 its comparatively weak peroxidative activity in the presence of hydrogen donors, when the [*Fe*$^{4+}$] complex is formed and undergoes partial autodestruction.

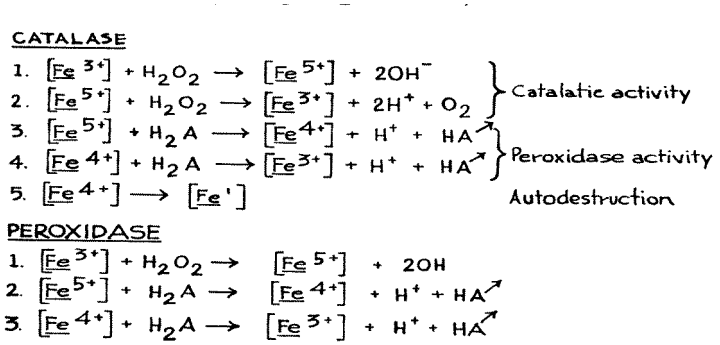

FIGURE 17. Mechanism of catalytic and peroxidative actions. [*Fe*] hmm iron, H_2A ascorbic acid.

A [Fe^{4+}] complex plays a normal role in the mechanism of peroxidase. Under certain conditions, catalase can thus act as peroxidase, but differs from peroxidase apparently in the properties of the [Fe^{5+}] and [Fe^{4+}] complexes. Only in catalase the [Fe^{5+}] complex reacts (directly or indirectly) with a second molecule of hydrogen peroxide. The [Fe^{4+}] complex of peroxidase is comparatively stable although some bile pigment is also formed (Kench, 1954).

The [Fe^{4+}] complex of catalase undergoes partial oxidation of its porphyrin ring. The verdohaem groups in liver catalase, and the rapid turnover of liver catalase iron (Theorell *et al*., 1951), are evidence for the peroxidative activity of liver catalase. No such evidence is available for erythrocyte catalase whose function is that of a safeguard against irreversible oxidation of haemoglobin to choleglobin by hydrogen peroxide (Foulkes and Lemberg, 1949).

Somewhat similar reactions of catalase occur in the presence of hydroxylamine or azide. Both are oxidized to a mixture of nitrous and nitric oxide (Foulkes and Lemberg, 1949*b*; Keilin and Hartree, 1954). The latter stabilises catalase in the ferrous form as [Fe^{2+}NO] complex. Keilin concludes from this and other experiments that the [Fe^{2+}] state is also passed during the normal action of catalase on hydrogen peroxide, but the matter is not yet clear.

Cytochromes. Lately our work has been mostly concerned with the cytochromes. The chain of electron passages from hydrogen donor to oxygen ends in a series of cytochromes, e.g. $b \rightarrow ? \rightarrow c \rightarrow a \rightarrow a_3$, where a_3 is almost certainly Keilin's cytochrome oxidase and Warburg's Atmungsferment. Whereas cytochrome *b* is derived from protohaem, and cytochrome c closely related to it (it may be considered an adduct of protein-cysteine to the vinyl groups of protohaem), cytochromes a_3, *a* and the bacterial cytochrome a_1 are derived from formylporphyrin. Cytochrome a_2 found in bacteria such as *Aerobacter*, *Azotobacter* or *Escherichia coli* is perhaps the terminal oxidase in these organisms; Barrett and Lemberg (1954) have recently isolated its haemin and shown that it is an iron complex of a chlorin, not a porphyrin. Porphyrin *a* or "cytoporphyrin" (Wargurg) has been obtained spectroscopically pure and in almost quantitative yield from heart muscle (Lemberg, 1953). This preparation largely excludes the formation of the cryptoporphyrins, which are artifacts, some derived from haem *a*, others from protohaem. The prosthetic groups of cytochrome oxidase (cytochrome a_3) and cytochrome *a* are generally assumed to be identical. Recently we have obtained two fractions of porphyrin *a* having exactly the same absorption spectrum, but differing in their extractability from ether by hydrochloric acid or phosphate buffer, as well as in their behaviour on cellulose or silica gel chromatographic columns. We have shown that these are mutually intraconvertible forms of porphyrin *a* and while it is not excluded that one is the prosthetic group of cytochrome a_3, the other of cytochrome *a,* our evidence so far does not support this assumption (Lemberg, 1955).

The structure of porphyrin *a* is not yet finally established. It has a formyl side chain and a side chain with a double bond conjugated with the porphin ring, probably on a pyrrole ring opposite to the one bearing the formyl. This, or another side chain, is a long alkyl group which increases the molecular weight without contributing to the colour. It appears difficult, however, to account for the two forms of porphyrin *a* on the basis of the present formulae.

The evidence for a long paraffinic side chain in porphyrin *a* is of particular interest. Both chlorophyll and cytochrome oxidase are contained in intracellular particles, the chloroplasts and mitochondria which possess a complicated internal structure of lipides and proteins. In the chloroplast the chlorophyll molecules form monolayers between protein and lipide layers (Fig. 18), with the long phytol tails sticking into the lipid layer which also contains the carotenoids (Wolken and Schwertz, 1953). In porphyrin *a* a similar structure is achieved by different means, not by esterification but by a long aliphatic side chain. Here is a whole new field opening for the biochemist, all that which lies between molecular and microscopic structure.

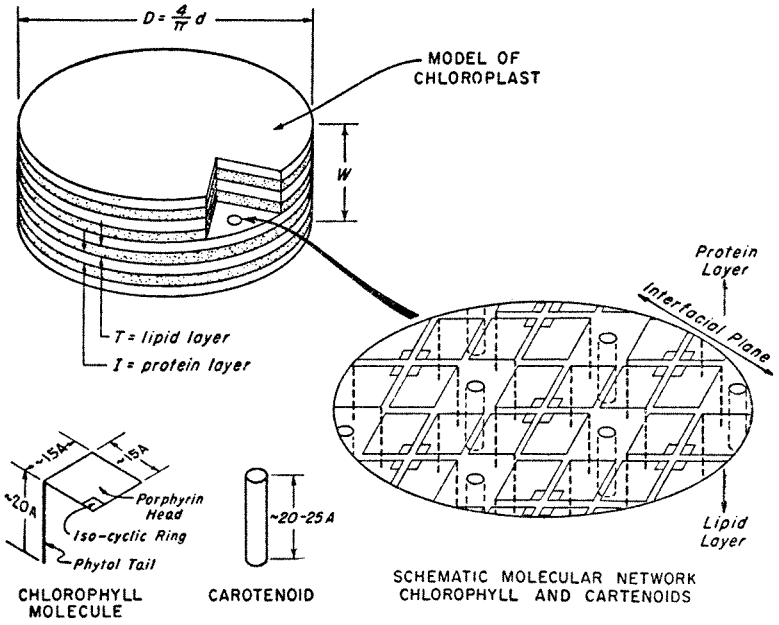

FIGURE 18. Chlorophyll monolayers in chloroplast (according to Wolken and Schwertz, 1953.)

Whereas usually flavoproteins (with isoalloxazine prosthetic groups) carry electrons from pyridine nucleotides to the cytochromes, Appleby and Morton (1954) have recently shown that cytochrome b_2 the lactic dehydrogenase of yeast, contains both haem and isoalloxazine groups on the same protein.

Biosynthesis of Porphyrins

The most important progresses in our field have been made during the last eight years in the exploration of the biogenesis of the haem compounds and of chlorophyll. H. Fischer had considered the uro- and coproporphyrins breakdown products of protohaem and products of carboxylation of protoporphyrin. As has been shown above, however, porphyrins are not in the normal way of haem breakdown, and there was accumulative physiological and pathological evidence (cf. Lemberg and Legge, 1949, pp. 593 ff., 628 ff.) that these free porphyrins in the body were formed in haemoglobin synthesis.

Uroporphyrin was the one found in the smallest amounts, so small that only recently Lockwood (1953, 1954) has been able to isolate pure uroporphyrin III from normal urine and to demonstrate that we excrete normally 10-30 μg. per day, one-fifth as much as coproporphyrin. Only in certain diseases (porphyrias), and in the fox squirrel (*Sciurus niger*) normally, is uroporphyrin excreted in milligramme amounts. The quantitatively insignificant or rare, in this instance uroporphyrin, in the instance of bile pigment formation biliverdin, had been considered of minor significance and the suggestion of Turner (1940) that uroporphyrin may be the primary porphyrin had found no acceptance. When in 1946 Rittenberg, Shemin and Bloch found in isotope experiments that N^{15} [*sic*] from glycine (Shemin and Rittenberg, 1946) and deuterium from acetate (Ponticorvo, Rittenberg and Bloch, 1949) were incorporated in the haem of haemoglobin by the nucleated erythrocytes of birds, I suggested a new hypothesis of porphyrin biogenesis in my lecture before the Adelaide Congress of A.N.Z.A.A.S., which was published three years later (Lemberg and Legge, 1949, p. 637). At that time it was a rather daring hypothesis, but it has meantime been proved almost entirely by American and English

workers. I have contributed nothing experimental to this development, but I believe that my 1949 discussions with Rimington and Neuberger, Rittenberg, Shemin, London, Bloch, Watson and Granick have hastened this development, and I am glad that one of my earlier pupils, John Falk, now Royal Society Foulerton Research Fellow at London University College, has been able to put what one may describe as the coping stone on this edifice.

My hypothesis was based on the following facts: The relationship between the side chains of the various porphyrins is such that the conversion of uro- to coproporphyrin

(4 CH_2CO_2H ---> 4 CH3) by decarboxylation

and that of copro- to protoporphyrin

(2 $CH_2CH_2CO_2H$ ----> 2 $CH{=}CH_2$) by oxidative decarboxylation are far more likely processes than the hitherto assumed inverse reactions (Fig. 19). The primary pyrrolic precursor thus should have the side chains of uroporphyrin, acetic and propionic side chains. Acetate enters the tricarboxylic acid cycle and in it becomes converted into α-ketoglutarate. Two molecules of α-ketoglutarate and two molecules of glycine can be expected to be condensed to such a precursor.

PORPHYRIN SYNTHESIS

Relation between porphyrins

Proto		Copro		Uro
2 $\cdot CH{=}CH_2$	←	2 $\cdot CH_2 \cdot CH_2 \cdot CO_2H$		2 $\cdot CH_2 \cdot CH_2 \cdot CO_2H$
2 $\cdot CH_2 \cdot CH_2CO_2H$		2 $\cdot CH_2 \cdot CH_2 \cdot CO_2H$		2 $\cdot CH_2 \cdot CH_2 \cdot CO_2H$
4 $\cdot CH_3$		4 $\cdot CH_3$	←	4 $\cdot CH_2 \cdot CO_2H$

FIGURE 19. Porphyrin biosynthesis. Relation between porphyrins. The arrows indicate the two reactions which do not necessarilyinvolve the side chains of the porphyrins themselves, butprobably those of monopyrrolic or dipyrrolic precursors.

Now here is the story as it stands today (Fig. 20). α-Ketoglutarate or succinyl coenzyme A formed in the citric acid cycle is condensed with one molecule of glycine to form α-amino-β-ketoadipic acid which is decarboxylated to δ-aminolaevulinic acid (Shemin and Russell, 1953). Two molecules of this are condensed to the pyrrolic precursor, porphobilinogen (Dresel and Falk, 1953). This substance was discovered by Waldenström in 1935 as a colourless precursor of porphyrin in the urine of patients with acute porphyria and assumed to be a dipyrrylmethane. Recently it has been isolated (Westall, 1952) and its structure as a monopyrrole established by Cookson and Rimington (1953). Finally Falk, Dresel and Rimington (1953) have shown that porphobilinogen is converted to uroporphyrin, coproporphyrin and the protoporphyrin of haem in the haemolysates of bird erythrocytes. The picture of these reactions as given in my 1946 lecture (Lemberg and Legge, 1949, p. 672) still remains essentially unaltered except that the assumed primary monopyrrolic precursor, and not the dipyrrylmethane is identical with porphobilinogen. In the role of porphobilinogen we have one more evidence, how a substance considered as the oddity of a specialist, can assume central importance, and how the study of pathological products can be fundamental for the explanation of normal physiological events. Inversely, there is now hope that our new insight will help us to

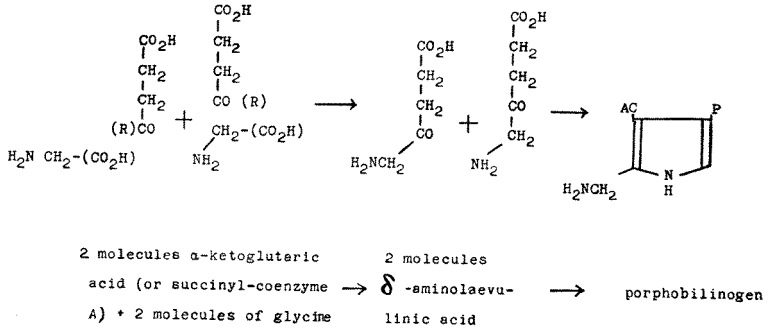

FIGURE 20. Porphyrin biosynthesis. Formation of monopyrrolic precursor. R represents -CO_2H group in α-ketoglutaric acid, -SR' group in coenzyme A. Groups eliminated during the reaction in parentheses.

find means to cure acute porphyria, a distressing and usually fatal disease not as rare as was previously believed.

The synthesis of chlorophyll proceeds along similar lines. The isotope experiments of Della Rosa, Altman and Salomon (1953) show that glycine and acetate are the primary precursors. Granick has discovered mutants of the green alga Chlorella which, instead of chlorophyll, contain the magnesium complex of protoporphyrin (Granick, 1948), that of vinylphaeoporphyrin a_5 (see Table 2) (Granick, 1950), or free highly carboxylated porphyrins (Bogorad and Granick, 1953).

We now begin to understand why the synthesis of porphyrins, so difficult for the organic chemist, is so easy a task for nature. A human adult forms no less than 80 g. of porphyrin annually and the amount of haemoglobin-porphyrin, alone, produced by mankind alone annually is about 160,000 tons. The production of chlorophyll is immeasurably greater and though the haematin enzymes are found in small concentrations, they are so widespread that the amounts of their prosthetic groups must also be very large.

These discoveries raise new problems with regard to biochemical evolution. The synthesis of the tetrapyrroles evidently presupposes the existence of a large part of the intermediary metabolism with its numerous enzymes so that it does no longer appear likely that their appearance on earth can have been so primeval as had been frequently assumed. If this is so, then almost all the metabolisn as we know it today, with photosynthesis and respiration, chlorophyll and haem enzymes, is far from primitive in terms of biochemical evolution, although we know from findings of porphyrins and chlorophyll derivatives in Silurian coals that it is at least 300 million years old. Moreover, we have the apparent contradiction that an oxidative cycle is necessary for the synthesis of the very catalysts which catalyse the oxidation. This vast complexity of the basis of life is often insufficiently appreciated by physicists as well as by people who speculate on the origin of life on earth.

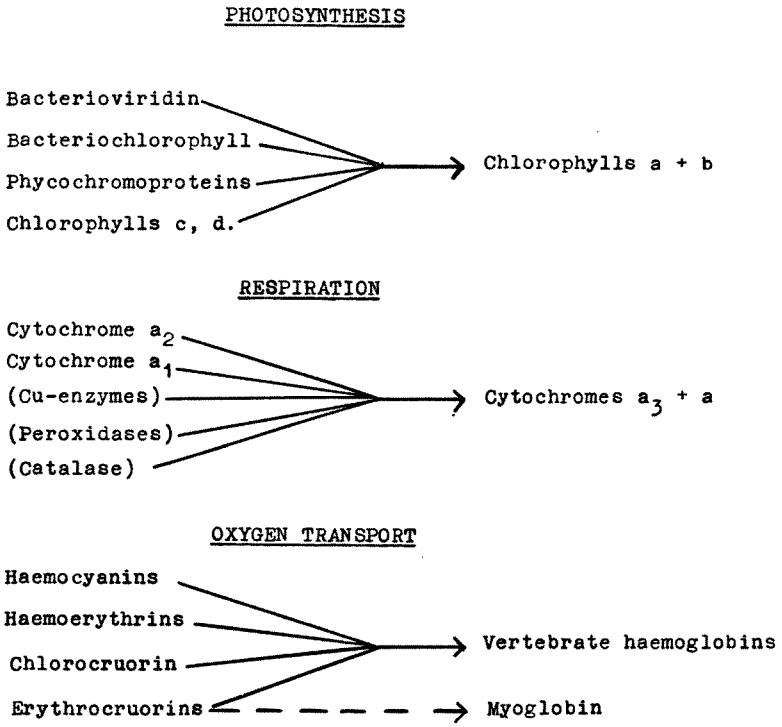

FIGURE 21. Convergent development

There is a tendency of convergent development which is displayed by the tetrapyrroles in nature. Nature appears to experiment with a variety of devices before it finally accepts one, which is then no longer modified (Fig. 21). Thus we find in the more primitive organism a variety of chlorophylls, and also phycochromoproteins, as catalysts in photosynthesis which in higher plants are replaced by the chlorophyll *a, b* system. In cellular respiration, a variety of cytochromes (a_1, a_2) and non-haem catalysts such as the copper enzymes, are finally replaced by the cytochrome a_3-*a* system. In a way, also, peroxidases and catalases become of minor significance with the development of this system. In oxygen transport, non-haem oxygen carriers, the Cu-containing haemocyanin, the iron-containing haemerythrins and the haemovanadins, as well as more primitive haemoglobins and chlorocruorin, are finally replaced by the vertebrate type of haemoglobin with four haem groups per molecule, while the perhaps more primitive monohaem protein answers a particular function as myoglobin in the red muscle cell. It appears that such compounds as haemoglobin have not been developed once but several times by Nature, in the same way in which organs, e.g. the placenta, have been constructed in different ways repeatedly.

Relation between Structure and Biological Fitness

We may now try to sum up the chemical features which we find to be the basis of biological fitness of the tetrapyrroles.

At first glance, it would appear difficult to reconcile the needs for a substance required for photosynthesis with those required for one functioning in cellular respiration, and again those for an oxygen carrier. The former requires a substance able to absorb sunlight and being able to hand on the energy of its activated molecule to the complicated mechanism by which the endergonic reaction $CO_2 + H_2O$ ---> $[CH_2O] + O_2$ is carried out. Absorption of light energy has no role in cellular respiration or oxygen transport. Again,

the ability to act as electron carrier between the substrates and oxygen in the respiratory chain appears to contradict the properties demanded for an oxygen carrier. In the first instance activation of oxygen and valency changes of the iron atom, in the latter lack of activation of oxygen and no valency change of iron.

Nevertheless, there are physicochemical features which make the porphyrin derivatives suitable for all these tasks. The first is the resonance structure, which at the same time is the basis of the absorption of visible light, helps the formation of stable metal complexes, and results in a most complicated interaction between the π-electrons of the porphin ring and the electrons of the metal.

The role of iron in cytochrome oxidase, the cytochromes, haemoglobin, peroxidases and catalase has been discussed. That of magnesium in chlorophyll is less certain. The original assumption of Willstätter that its role is the binding of carbon dioxide has been disproved. It now appears much more likely that it is water that is bound on the magnesium atom of chlorophyll, and that the first reaction of photosynthesis, written schematically

$$H_2O + h\nu \dashrightarrow OH + H,$$

takes place with water thus bound. Chlorophylls form hydrates, as has been demonstrated by the change of absorption and fluorescence which they undergo if water is added to their solutions in water-free hydrocarbons (Livingston and Well, 1952).

The importance of the resonance structure is, however, still larger. If a monovalent electron change is to be connected with the usual divalent hydrogen changes of organic substrates, or with the monovalent changes between oxygen and water, or hydrogen peroxide and water, without the formation of indiscriminately reactive monovalent radicals, such radicals must be resonance-stabilised. Radicals such as OH, O_2H and H have been freely postulated by physicochemists but I doubt whether such almost indiscriminately reactive and freely diffusible radicals could be subjected to the fine control (homeostasis) characteristic of every living cell and could find a place in normal biological reactions. In fact such radicals cause the destruction by γ-radiation. Nature uses the device of letting such reactions take place on large resonance stabilised molecules, whose monovalent radicals are less reactive and less or non-diffusible, and which, moreover, are held in position by their linkage to proteins in a well organised chain in special cell organs, such as the mitochondria or chloroplasts. It is thus not accidental that so many of the catalytically important molecules of the cell are coloured, even where colour *per se* is no requisite. Evidence for such resonance-stabilised radicals in the tetrapyrrole field is still largely circumstantial. There is no reliable evidence for the role of a mono-dehydrochlorophyll in photosynthesis or for reversible alteration of the porphyrin ring in haematin enzymes. We must go one step further and consider the whole porphyrin-iron-protein complex. Haemin is a 1000 times better catalatic catalyst than iron, but catalase is 1000 million times more effective. No simple haem compound has the ability of reversibly combining with oxygen, which is the functional characteristic of haemoglobin. This requires a specific type of protein, globin. The iron of protohaem behaves quite differently when it is combined with the respective proteins in haemoglobin, cytochrome *b*, catalase or horse radish peroxidase. We have no less than five different ways of behaviour of haem iron which depend mainly on the protein and, with the possible exception of cytochrome oxidase, not on the side chains of the porphin ring. In haemoglobins and myoglobins: $[Fe^{2+}] + O \rightleftharpoons [Fe^{2+}O_2]$; in cytochrome

oxidase: [Fe^{2+}] + O_2 ------> [Fe^{3+}] ; in non-autoxidisable cytochromes: [Fe^{2+}] $\rightleftharpoons$ [Fe^{3+}]+e. About the reactions of catalases and peroxidases, see Figure 17.

It will require a far greater knowledge than we possess today of protein structure, of the type of linkage between prosthetic group and protein, and of quantum mechanics, to understand the complex interactions between π-electrons of the porphyrin, electrons of the iron and the influence of the protein bound to the metal. It is the protein which determines the finest adaptations and variations, e.g. those between haemoglobins and myoglobins, and still finer ones between the different haemoglobin of various species, and even in one and the same species, between several haemoglobins, such as foetal and adult, or normal adult and a variety of pathological haemoglobins. This, however, leads us out of the field which I have proposed to discuss into the different one of protein specificity, and I must restrict myself to having pointed out the connection.

We still know far too little, both of biological function and of chemical structure, and particularly about the relations between these two, to see more than a faint glimmer. But this glimmer is enough to let us stand in awe of the incredible complexity and the degree of fitness in this complexity. Perhaps Voltaire's sneer about this "best of all possible worlds" was hardly less one-sided than the all too complacent idea of an order of absolute infallibility in Nature. Surely the truth lies somewhere in between "what a scene of gratification and pleasure" and "Nature red in tooth and claw", between Henderson's "coordinated fitness of animate and inanimate world" and "struggle for life in a hostile environment ".

I cannot close without remembering in gratitude some of my teachers: Heinrich Biltz, to whom I owe most of the little skill I possess as an experimentalist, and an education to patience and perseverance; Karl Freudenberg, who showed me the wide possibilities for the organic chemist in the exploration of natural products; Sir Frederic Gowland Hopkins, David Keilin and other Cambridge friends, who turned my attention from structural to metabolic and functional problems and thus began the conversion of an organic chemist to a biochemist.

My collaborators, many unnamed here, have borne more than their share of troubles in our adventures but have, I hope, also participated in the joys of exploration. Without the support of the hospital authorities, and in particular of the National Health and Medical Research Council, my researches could not have been carried out.

I thank the Royal Society of N.S.W. for the honour they have conferred upon me by entrusting me with this lecture, and I hope that I have shown some lines from my work which may lead into the future.

References

Appleby, C.A., and Morton, R.K., 1954. *Nature,* **174**, 749.
Barrett, J., and Lemberg, R., 1954. *Nature,* **173**, 213.
Bogorad, L., and Granick, S., 1953. *J.biol. Chem.,* **202**, 793.
Chance, B., 1948. *Nature,* **161**, 914.
Cookson, G.H., and Rimington, C., 1953. *Nature,* **171**, 875.
Della Rosa, R.J., Altman, K.I., and Salomon, K., 1953. *J. biol. Chem.,* **202**, 771.
Dresel, E.I.B., and Falk, J.E., 1953. *Nature,* **172**, 1185.
Duysens, L.N., 1951. *Nature,* **168**, 548.

Falk, J.E., Dresel, E.I.B., and Rimington, C., 1953. *Nature,* **172**, 292.
Fischer, H., and Orth, H., 1937. *Die Chemie des Pyrrols,* **2**, 1. Akad. Verlagsgesellschaft, Leipzig.
Foulkes, E.C., and Lemberg, R., 1948. *Austral. J. Exp. Biol. Med. Sci.,* **26**, 307.
Foulkes, E.C., and Lemberg, R., 1949*a*. *Proc. Roy. Soc. B.,* **136**, 435.
1949*b*. *Enzymol.,* **13**, 302.
Foulkes, E.C., Lemberg, R., and Purdom, P., 1951. *Proc. Roy. Soc. B.,* **138**, 386.
George, P., 1952. *Biochem. J.,* **52**, xix.
1953. *Biochem. J.,* **54**, 267.
Granick, S., 1948. *J. biol. Chem.,* **175**, 333.
1950. *J. biol. Chem.,* **183**, 713.
Hackman, R.H., 1952. *Arch. Biochem. Biophys,* **41**, 166.
Haxo, F.T., and Blinks, L.R., 1950. *J. Gen. Physiol.,* **33**, 389.
Keilin, D., and Hartree, E.F., 1954. *Nature,* **173**, 720.
Kench, J.E., 1954. *Biochem. J.,* **56**, 669.
Kitasato, Z., 1925. *Acta Phytochimica (Japan),* **2**, 75.
Küster, W., and Deihle, H., 1913. *Z. physiol. Chem.,* **82**, 463.
Lemberg, R., 1930. *Lieb. Ann.,* **477**, 195.
1931. *Lieb. Ann.,* **488**, 74.
1932. *Lieb. Ann.,* **499**, 25.
1935. *Biochem. J.,* **29**, 1322.
1943. *Austral. J. Exp. Biol. Med. Sc.,* **21**, 239.
1953. *Nature,* **172**, 619.
1955. In "Biochemistry of " Nitrogen", *Suomalainen Tiedeakatemia,* Helsinki, p. 165.
Lemberg, R., and Bader, G., 1933. *Lieb. Ann.,* **505**, 151.
Lemberg, R., and Barcroft, J., 1932. *Proc. Roy. Soc. B.,* **110**, 362.
Lemberg, R., and Foulkes, E.C., 1948. *Nature,* **161**, 131.
Lemberg, R., and Legge, J.W., 1949. " Hematin Compounds and Bile Pigments." Interscience, New York.
Lemberg, R., Norrie, M., and Legge, J. W., 1939. *Nature,* **144**, 551.
Lemberg, R., and Wyndham, R. A. *Biochem. J.,* **30**, 1147.
Linstead, R.P., 1934. *J . Chem. Soc.,* 1016.
1953. *J. Chem. Soc.,* 2837.
Livingston, R., and Well, S., 1952. *Nature,* **170**, 750.
Lockwood, W.H., 1953. *Austral. J. Exp. Biol. Med. Sc.,* **31**, 453.
Lockwood, W.H., and Bloomfield, B., 1954. *Austral. J. Exp. Biol. Med. Sc.,* **32**, 733.
Morell, D., 1953. *Austral. J. Exp. Biol. Med. Sc.,* **31**, 567.
Ponticorvo, L., Rittenberg, D., and Bloch, K., 1949. *J. biol. Chem.,* **179,** 839.
Robertson, J.M., 1935. *J. Chem. Soc.,* 615.
Shemin, D., and Rittenberg, D., 1946. *J. biol. Chem.* **166**, 621.
Shemin, D., and Russell, C.S., 1953. *J. Amer. Chem. Soc.,* **75**, 4873.
Theorell, H., Beznak, M., Bonnichsen, R., and Paul, K.G., 1951. *Acta Chem. Scand.,* **5**, 445.
Turner, W.J., 1940. J. Lab. Clin. Med., 26, 323.
Waldenström, J., 1935. *Dtsch. Arch. klin. Med.,* **178**, 38.
Warburg, O., 1932. *Z. angew. Chem.,* **45**, 1.
Warburg, O., and Negelein, E., 1930. *Ber. Dtsch. Chem. Ges.,* **63**, 1816.
Westall, R.G., 1952. *Nature,* **170**, 614.
Wolken, J.J., and Schwertz, F.A., 1953. *J. Gen. Physiol.,* **37**, 11.
Wood-Jones, F., 1939. "The Spirit of Adventure," in " Life and Living ". Routledge, London.