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On the Respiratory Function of Haemoglobin

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Contents

Hardly any molecule has attracted the interest of as many scientific disciplines as has haemoglobin. Contributions from physicists, chemists and biologists have combined to bring about a full understanding of the relationship between its structure and function. In particular, the finding that red cell metabolism and haemoglobin function are interdependent has provided important biological insights that have transformed the red cell from a seemingly inconspicuous biological unit into a model demonstrating several fundamental biological principles.

It is the aim of this article to summarise current knowledge about the physiological properties of haemoglobin in transport of oxygen and carbon dioxide in the blood, and to relate this to its molecular structure.

I. The Chemistry of Haemoglobin

Every vertebrate haemoglobin known is an oligomeric protein. They consist of four polypeptide subunits (protomers) each of which contains an oxygen binding

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protohaem which is a chelate complex of protoporphyrin with a bivalent iron atom. Each two of the polypetide subunits has an identical amino acid sequence. In human haemoglobin A (A for adult) the two identical α-subunits consist of 141 amino acid residues, whereas the two β-subunits are built up from 146 residues. Besides haemoglobin A ($\alpha_2\beta_2$) there are at least two more biologically important human haemoglobins: haemoglobin F (F for foetal) which has the structure $\alpha_2\gamma_2$ and haemoglobin A_2 ($\alpha_2\delta_2$). HbF is the major oxygen carrying pigment during the last two thirds of gestation and HbA_2 is a minor component in the blood of adults (about 2.5 per cent of total Hb).

Both HbF and HbA_2 have the same α-subunits as HbA. Their γ- and δ-subunits, however, differ from the β-subunits in the *sequence of the amino acid residues* (SCHROEDER, 1963; BRAUNITZER et al., 1964; SCHROEDER and JONES, 1965). All the various subunits possess a certain sequence homology, particularly if some gaps are introduced. This sequence homology can be interpreted as the result of gene duplications during phylogeny and the conclusion can be drawn that the γ-subunits were the first to develop independently of the α-subunits, followed by the β- and the δ-subunits (ZUCKERKANDL and PAULING, 1962; BRAUNITZER, 1967).

The *secondary structure* of all subunits shows a high content of α-helix comprising about 80 per cent of all amino acid residues. The helical sections are called helices A to H, starting at the N-terminus of the subunit. The nonhelical segments or corners are denoted according to the adjoining helices, e.g., AB, BC etc. Residues within each segment are numbered from the amino end (PERUTZ, 1965). Helix D is deleted in the α-subunits.

The *three dimensional folding* of the polypeptide subunits, like their secondary structure, was elucidated by means of X-ray diffraction analyses of crystals of haemoglobin and myoglobin, the latter being a monomeric haemoprotein found in muscle cells (KENDREW et al., 1960; PERUTZ et al., 1960; MUIRHAED and PERUTZ, 1963; PERUTZ et al., 1964; PERUTZ, 1965; MUIRHAED et al., 1967; PERUTZ et al., 1968; MUIRHAED and GREER, 1970; BOLTON and PERUTZ, 1970). The most striking feature of the tertiary structures of the different subunits is their close resemblance in spite of rather large differences in primary structure. The majority of the amino acid side chains (R groups) protruding into the interior of the subunits are nonpolar, or hydrophobic, in character so that the molecule is held together by numerous hydrophobic interactions. The polar R groups, on the other hand, are mainly in contact with the solvent where the charged side chains are exposed to the water phase. This particular distribution of hydrophilic and hydrophobic R groups explains the solubility of haemoglobin, even at the very high concentration found in red cells.

The *haem group* is embedded in a hydrophobic pocket between helices E and F. The only covalent bond between the haem group and the R groups forming the wall of the haem pocket is between histidine F (8) and the haem iron, whilst most interactions (about 60) are again hydrophobic in nature. The invariance of the residues surrounding the haem group suggests that almost all of them are essential for a proper function of the haemoglobin molecule (PERUTZ et al., 1968).

The hydrophobic environment of the haem group is thought to explain the unusual stability of the oxygen-haemoglobin complex. It was suggested that the reversible binding of oxygen to the haem group is due to the low dielectric constant

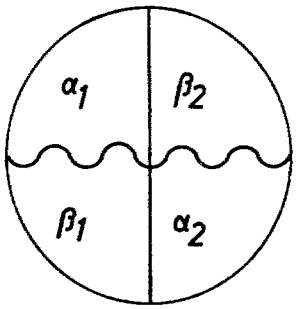

Fig. 1. Schematic representation of the assembly of α- and β-subunits in haemoglobin

of water in the hydrophobic environment of many aliphatic hydrocarbon R groups and aromatic rings (WANG, 1958; WANG et al., 1958). By embedding a ferrous haem-imidazole complex in a matrix of polystyrene, which is analogous to the hydrophobic environment of the haem pocket, a model compound can be obtained which is capable of reversible combination with oxygen (WANG, 1958). This experiment seems to offer proof of the conclusion that the low dielectric constant of water in the haem pocket prevents the superoxide anion, which is probably formed at the 6th coordination site of the haem iron (see Reaction 1), from dissociation from an oxidized haem iron (WEISS, 1964; VIALE et al., 1964; WITTENBERG et al., 1970).

$$\begin{array}{cc} \text{N} \quad \text{N} & \text{N} \quad \text{N} \\ \diagdown \diagup & \diagdown \diagup \\ \text{Globin—Fe}^{++} + \text{O}_2 \rightleftharpoons & \text{Globin—Fe}^{+++} \cdot \text{O}_2^- \\ \diagup \diagdown & \diagup \diagdown \\ \text{N} \quad \text{N} & \text{N} \quad \text{N} \\ \text{Deoxyhaemoglobin} & \text{Oxyhaemoglobin} \end{array} \tag{1}$$

As oxyhaemoglobin becomes deoxygenated when travelling through the tissue capillaries, Reaction (1) proceeds from right to left because the superoxide anion is held back in the haem pocket where the dielectric constant of water is only slightly higher than that of benzene (PERUTZ, 1965), whilst molecular oxygen is perfectly free to leave the pocket.

The *quaternary structure* of the tetrameric haemoglobin is maintained by a number of contacts between α- and β-subunits, most of which are hydrophobic. One can sketch the assembly of α- and β-subunits in the following way (Fig. 1) which represents the view along the dyad axis of the molecule. Between the subunits α_1 and β_1 the contacts consist of thirty-four residues, whereas the contact between α_1 and β_2 involves nineteen residues belonging to helices C, F and G. Upon ligand binding large structural changes are observed at this latter contact which led to the conclusion that it may be important from a functional view point (PERUTZ et al., 1968; MUIRHAED and GREER, 1970). Support for this idea comes from the functional properties of mutant haemoglobins. Among other things, a diminished haem-haem interaction has been reported in all cases where the $\alpha_1\beta_2$ contact is affected (PERUTZ and LEHMANN, 1968). It should be noticed, however, that the $\alpha_1\beta_2$ contact cannot be thought of simply as a pathway, along which

information is carried from one haem group to another, but rather as a functional unit which must be seen in relation to other specific contacts between the subunits. The importance of some of these contacts for the functional properties of haemoglobin were extensively discussed by PERUTZ (1970), and will be dealt with in the following paragraph.

II. Allosteric Effects in Haemoglobin

A. General Considerations

The physiologically most important properties of haemoglobin are the cooperativity of oxygen binding and the influence of hydrogen ions, carbon dioxide and organic phosphate compounds on the affinity of haemoglobin for oxygen. The basis for comprehending these properties is the fact that haemoglobin undergoes a structural transition when oxygen and other haem ligands (e.g. CO, NO) are bound or removed. This structural change was observed as early as 1938 by HAUROWITZ who states: „Aus diesen Versuchen geht deutlich hervor, daß die Bindung des Sauerstoffs an das Hämoglobin mit einem Umbau des Krystallgitters verbunden ist. Er ist vermutlich auf eine Änderung der zwischen den Hämgruppen wirksamen Kräften zurückzuführen". ("From these experiment it follows clearly that the binding of oxygen to haemoglobin is accompanied by a rearrangement of the crystal lattice. It is probably attributable to a change in the forces acting between the haem groups.") This rearrangement of the crystal lattice occurring when ligands are bound or removed, was later elucidated in striking detail by MAX PERUTZ and his colleagues in Cambridge (PERUTZ et al., 1960; MUIRHAED and PERUTZ, 1963; PERUTZ, 1965; MUIRHAED et al., 1967; PERUTZ et al., 1968; MUIRHAED and GREER, 1970; BOLTON and PERUTZ, 1970). The idea that the change of molecular shape of haemoglobin which occurs upon ligand binding is responsible for its functional properties, was first theoretically developed by WYMAN and ALLEN (1951) and later extended by WYMAN (1963, 1967). WYMAN and ALLEN clearly foresaw that both the interaction between the haems of haemoglobin and the Bohr effect are due to conformational changes of the molecule as a whole, and that a number of chemical differences between oxy- and deoxyhaemoglobin (BENESCH and BENESCH, 1963) are connected to the same phenomenon.

The term *allosteric* (from greek ἄλλος στέρς, different appearance) was introduced into biological terminology by MONOD and JACOB (1961) to describe the effect of an inhibiting or activating agent on the action of an enzyme in terms of the structural alterations of the protein (allosteric transition), induced by the binding of a ligand. Later this concept was extended and refined (MONOD et al., 1963) and resulted finally in the formulation of a specific allosteric model (MONOD et al., 1965). With regard to haemoglobin the model requires the existence of a molecular conformation with a low oxygen affinity (T-conformation, T for tense) which is in equilibrium with a conformation where the oxygen affinity is high (R-conformation, R for relaxed). All substances (allosteric effectors) which affect the equi-

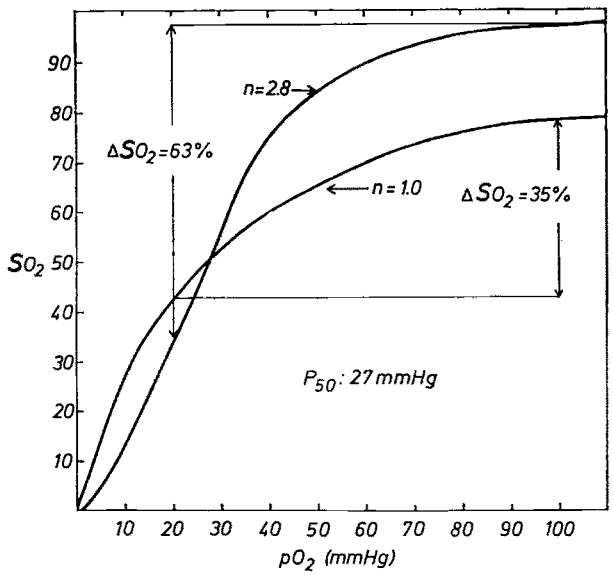

Fig. 2. Cooperative ($n=2.8$) and non-cooperative ($n=1.0$) oxygen haemoglobin equilibrium curves with the same oxygen affinity. ΔS_{O_2} is the amount of oxygen which can be extracted from the blood when the partial pressure of oxygen falls from 100 mm Hg (in the lungs) to 20 mm Hg (in the coronary sinus)

librium between these two conformations, i.e., the allosteric transition of the protein, will correspondingly affect the oxygen affinity. It is noteworthy to recall, that, as early as 1961, NIESEL described a model for the reaction of haemoglobin with oxygen which contains many of the characteristic features of the allosteric model of MONOD et al. (1965).

B. The Oxygen Haemoglobin Equilibrium Curve

The relationship between the oxygen pressure and the saturation of haemoglobin with oxygen is shown in Fig. 2. It is immediately apparent that, unlike the monomeric myoglobin, the tetrameric haemoglobin binds oxygen in a cooperative fashion which is expressed in the sigmoidal shape of the curve (BOHR, 1903). This cooperativity is brought about by the fact that oxygen binding to certain sites in haemoglobin increases the oxygen affinity of the remaining ones. A quantitative estimate for cooperativity is given by the exponent n in the equation put forward by HILL (1910):

$$Y = \frac{K \cdot p^n}{1 + Kp^n} \tag{2}$$

in which Y, K and p denote the oxygen saturation, the dissociation constant and the oxygen pressure, respectively. The exponent "n" was originally (HILL, 1910) assigned to the number of subunits which combine to form a polymeric haemo-

globin. However, "n" is now thought to represent the minimal estimate of the number of haem groups which interact upon ligand binding, i.e. for the *haem-haem interaction* (PAULING, 1935; WYMAN, 1948). Depending on the experimental conditions "n" is found to be about 2.8 for all normal tetrameric haemoglobins at neutral pH in an oxygen saturation range between 20 and 80 per cent. A very low degree of haem-haem interaction ($n \simeq 1$) should be expected if a given haemoglobin does not undergo an allosteric transition on ligand binding, and this has indeed found to be the case. Haemoglobin H, a haemoglobin composed only of β-subunits, behaves functionally almost like monomeric myoglobin (BENESCH et al., 1961), and its crystal structure is independent of the absence or presence of ligands (PERUTZ and MAZZARELLA, 1963).

The *physiological meaning of cooperative oxygen binding* in haemoglobin can be assessed immediately on the basis of the curves shown in Fig. 2. It can be seen that if haemoglobin exhibits normal cooperativity ($n=2.8$), the amount of oxygen which may be extracted from it as the partial pressure of oxygen falls from that of arterial blood to that of venous blood is almost twice as much as that extracted if it behaves like a monomer with no cooperativity ($n=1.0$). It should be noted that this large difference in the availability of oxygen is mainly due to the reduction of oxygen saturation at the arterial pO_2 which would lead to a much reduced oxygen content in the arterial blood. Although a cooperative oxygen binding seems to be advantageous when the oxygen affinity is normal, or even reduced, it becomes detrimental when the oxygen affinity is elevated. When there is a high oxygen affinity the lesser inflection of the curve promotes the unloading of oxygen, as can be seen from the functional properties of some abnormal haemoglobins (BELLINGHAM, 1972).

The cooperative binding of haem ligands with its obvious physiological implications has evoked the theoretical interest of many workers and has resulted in the formulation of a substantial number of models other than the allosteric one. Unfortunately, within the limits of the present article, it is only possible to discuss the main alternative to the "allosteric" model put forward by KOSHLAND et al. (1966) and named the "induced fit" model. Many other models have been discussed by NIESEL (1961) and more recently by WHITEHAED (1970) and ANTONINI and BRUNORI (1971).

The gist of the model of KOSHLAND and his colleagues is that oxygen binding to one subunit in the tetrameric haemoglobin causes a conformational change solely in that subunit which in turn will *induce* an alteration of the tertiary structure of a neighbouring subunit. This "induced fit" leads then to a higher reactivity towards the next oxygen molecule to be bound which, once it is in place, will induce another fit and so forth. The main difference between the "allosteric" and the "induced fit" model is based predominantly on different assumptions about the path of the conformational changes. Whereas the allosteric transition from the low-affinity to the high-affinity quaternary structure is thought to occur in an all or none fashion without tertiary-tertiary interactions, the induced-fit transition is supposed to take place sequentially by tertiary-tertiary interactions only, without any influence of quaternary constraints.

As was to be expected, there was and is still disagreement in respect to the validity of the two models. EDELSTEIN (1971), for example, in analyzing oxygen

binding curves obtained under various conditions, came to the conclusion that only the two-state model could account for the functional behaviour of haemoglobin. Similar inferences were reached by HOPFIELD et al. (1971) and by SHULMAN et al. (1972) on the basis of analyses of kinetic data. Further support for the two-state model came from measurements of the binding of a spin-labeled triphosphate to haemoglobin with the additional assumption of differences in the functional behaviour of α- and β-subunits (OGATA and MCCONNELL, 1971; 1972a, b).

The induced-fit model, on the other hand, received support from the analyses of haemoglobin equipped with a spinlabel at the SH-group of cysteine F 9 (93) β which was studied at different levels of ligand binding (OGAWA and MCCONNELL, 1967). Also, the fact that a haemoglobin with irreversibly liganded α-subunits shows cooperativity (between the β-subunits) was taken as a case where the "induced fit" applies (HABER and KOSHLAND, 1971). Another example of subunit allostery is found in haemoglobin Iwate, a mutant haemoglobin which is frozen in the quaternary T-structure. In this haemoglobin the α-subunits are oxydized, and therefore unable to react with oxygen or carbon monoxide. Nevertheless, the protein exhibits distinct interactions between the β-subunits which are independent of changes of the quaternary structure (GERSONDE et al., 1973).

If one tries to view this situation from a distance, it becomes quite obvious that there are experimental situations which can be adequately described by one or the other model, particularly if certain modifications are introduced to augment the degrees of freedom for a given model. It seems to be unlikely, therefore, that the general validity of any model can be assessed by showing that it can fit the data of more or less limited experimental conditions. Furthermore, there are now experimental results accumulating which indicate that partially liganded haemoglobin may have a structure (tertiary and/or quaternary) different from that of oxy- and deoxyhaemoglobin (HUESTIS and RAFTERY, 1972; CASSOLY and GIBSON, 1972; OLSON and GIBSON, 1973a) and that apparently even the structure of plain deoxyhaemoglobin and oxyhaemoglobin changes, depending on the solvent conditions (DEAL et al., 1971; MCDONALD and NOBLE, 1972; OLSON and GIBSON, 1973b; HENRY and BANERJEE, 1973). Thus, at present it seems to be safe to rely on models which are formulated in a more general way (HERZFELD and STANLEY, 1972) leaving enough room for the complexity of interactions between haemoglobin and ligands.

A very detailed mechanism on the nature of the haem-haem interaction was proposed by PERUTZ (1970, 1972) on the basis of stereochemical observations. PERUTZ found the deoxy quaternary structure of haemoglobin to be stabilized by six specific salt-bridges not only between the α- and β-subunits and the two α-subunits but also within the β-subunits. These salt-bridges hold the molecule in a state of low oxygen affinity (T state in the sense of MONOD et al., 1965). As oxygen is bound to the subunits, these salt-bridges are sequentially broken until the molecule "clicks" into the relaxed oxy conformation which has an oxygen affinity 100–200 times higher than has deoxyhaemoglobin. The major change in the quaternary structure seems to occur after the third ligand is bound to haemoglobin (ROUGHTON et al., 1955, 1972; TYUMA et al., 1971; MACQUARRIE and GIBSON, 1971; SALHANY et al., 1972). This rather large change of the quaternary

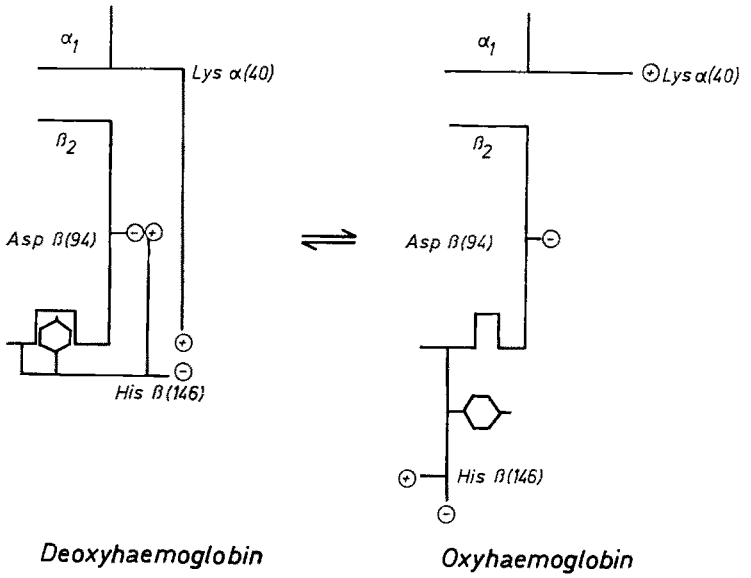

Fig. 3. Position of the penultimate tyrosine of the β-subunits in deoxyhaemoglobin and oxyhaemoglobin. (After PERUTZ, 1970)

structure is induced by a comparatively minute movement of the iron atom when it changes its spin state from high spin to low spin upon ligand binding. In deoxyhaemoglobin the haem iron is in a state of high spin and found to be displaced out of the haem plane by 0.7–0.8 Å. As oxygen is bound, the haem geometry changes, inasfar as the iron moves back into the haem plane (HOARD, 1966; BANERJEE et al., 1969; PERUTZ, 1970). This movement induces a shift of helix F towards the centre of the molecule in the direction of helix H.

In deoxyhaemoglobin helices F and H of each subunit form a pocket which accomodates its penultimate tyrosine HC 2 (140) α and HC 2 (145) β. The movement of helix F narrows the pocket and causes the tyrosine's expulsion (Fig. 3), pulling the C-terminal residues with it and thereby breaking some of the salt-bridges which hold the molecule in the tense deoxy conformation. The breaking of the salt-bridges leads then to a rearrangement of the quaternary conformation and a consecutive increase in oxygen affinity. Clearly, the penultimate tyrosines act as levers which magnify many times the small movement of the iron atom upon ligand binding, giving rise to cooperative ligand binding (KILMARTIN and HEWITT, 1971). A very similar mechanism has been described for a monomeric type of insect haemoglobin in connection with the liberation of protons on ligand binding (SICK et al., 1972).

Another important point of the stereochemical model concerns the different oxygen affinity of the α- and β-subunits. Since the haem pockets of the α-subunits have more room next to the haem than have the β-subunits, PERUTZ (1970) proposed that the α-subunits should take up oxygen preferentially. The experimental evidence obtained so far points towards either the α- (HAYASHI et al., 1968; LINDSTROM and HO, 1972; HUESTIS and RAFTERY, 1972; HENRY and CASSOLY, 1973) or

the β-subunit (LINDSTROM et al., 1971; GIBSON, 1973; OLSON and GIBSON, 1973a, b) as the faster reacting species, depending mainly on the type of ligand studied. It is significant that interactions between α- and β-subunits are apparently transmitted from the β-subunit to the α-subunit and not the other way round (BANERJEE et al., 1973). Furthermore, ligand binding to the β-subunits only leads to a more pronounced alteration of the molecular shape towards the oxy conformation than does ligand binding to only the α-subunits (BAUER et al., 1973).

C. The Binding of Carbon Dioxide to Haemoglobin

On the way from the tissues back to the lungs haemoglobin is involved in the transport of carbon dioxide in two ways: firstly by the direct combination of carbon dioxide with haemoglobin, and secondly by the oxygen-linked uptake and release of protons. The direct combination of carbon dioxide with haemoglobin was discovered by HENRIQUES (1928, 1929). By the work of others it quickly became apparent (FERGUSON and ROUGHTON, 1934a, b; FERGUSON, 1936; STADIE and O'BRIEN, 1937) that the reaction between haemoglobin and carbon dioxide belongs to the general carbamate type (SIEGFRIED, 1905; FAURHAULT, 1925) in which carbon dioxide reacts with uncharged amino groups according to the following reaction scheme:

$$\begin{aligned} &R\text{–}NH_3^+ \rightleftharpoons R\text{–}NH_2 + H^+ && a \\ &R\text{–}NH_2 + CO_2 \rightleftharpoons R\text{–}NHCOOH && b \\ &R\text{–}NHCOOH \rightleftharpoons R\text{–}NHCOO^- + H^+ && c \end{aligned} \qquad (3)$$

Among all the residues which in principle are capable of forming carbamate, the terminal α-amino groups of both α- and β-subunits have long been suspected as the most likely candidates at physiological pH. It will be noted from the reaction scheme that carbon dioxide does not react with the protonated form of the amino group ($R\text{–}NH_3^+$). From this consideration it follows that the ε-amino groups of the numerous lysine residues, which have a pK of about 10.5, cannot participate significantly in the carbamate reactions at physiological pH. Provided that step (b) in the above reaction is the same for both α- and ε-amino groups, it follows that most carbon dioxide molecules form carbamate at the α-amino groups which have a pK value of about 7.0–8.0 (ROSSI-BERNARDI and ROUGHTON, 1967; ROUGHTON, 1970; KILMARTIN and ROSSI-BERNARDI, 1971).

This reasoning was later verified directly in experiments with haemoglobin specifically blocked with cyanate at the α-amino groups of either the α- or the β-subunits (KILMARTIN and ROSSI-BERNARDI, 1969, 1971). KILMARTIN and ROSSI-BERNARDI showed by various experiments including differential titration, carbon dioxide binding and oxygen haemoglobin equilibrium curves that, indeed, the terminal α-amino groups are equally and solely responsible for the carbamate reaction at physiological pH. It should be noted, however, that the reaction of the α-amino groups with cyanate of the α-subunits is accompanied by a rather large increase of the oxygen affinity and that the blocking of the corresponding groups in the β-subunits leads to a decrease in cooperativity. This is to say that blocking of one or the other site in haemoglobin may well lead to significant changes in

properties of the molecule without corresponding structural changes detectable by X-ray crystallography. Whilst the conclusions of KILMARTIN and ROSSI-BERNARDI on the location of the binding site of carbamate seem to be unimpeachable, the relative contribution of α- and β-subunits to the reaction should be reexamined with different blocking agents in order to rule out the possibility that the chemical manipulation at one pair of subunits alters the carbon dioxide reactivity of the other pair.

Under physiological conditions the amount of carbamate formed by the haemoglobin molecule depends upon at least three variables: pH, the saturation of haemoglobin with oxygen and the concentration of organic phosphate esters.

The *influence of pH* on the carbamate reaction becomes immediately clear from the reaction scheme. An increase of pH increases the number of unprotonated amino groups (step a) and, at the same time, shifts step c to the right. Therefore, an increase in pH leads to an increase in carbon dioxide affinity through both reactions.

The *effect of oxygen binding* on the carbamate reaction is not that easy to understand. It was not until the late sixties that ROSSI-BERNARDI and ROUGHTON (1967) and FORSTER et al. (1968) verified the assumption of FERGUSON and ROUGHTON (1934a, b) that *at constant pH* less carbamate is formed by oxygenated haemoglobin solutions than by deoxygenated ones. This was the proof that the carbamate is truly linked to oxygen binding and not, as WYMAN (1948) had proposed, just linked to the changes in pH accompanying changes in the state of ligation of the haemoglobin.

Evidently, the reduced carbamate formation of fully liganded haemoglobin must be connected to the characteristic conformational change, but the available information on this point is puzzling. According to PERUTZ (1970) one of the pairs of salt-bridges stabilizing the deoxy conformation is formed between the α-amino groups of Val NA (1) and the carboxyl groups of Arg HC3 (142) of the α-subunits. These links are broken in oxyhaemoglobin, whereas the α-amino groups of the β-subunits seem to be free, irrespective of the state of ligation. From this one would expect the carbon dioxide affinity of oxyhaemoglobin to be actually higher than that of deoxyhaemoglobin, which is not the case. One explanation is that in deoxyhaemoglobin the negatively charged carbamate groups form strong secondary salt-links with neighbouring positively charged R groups; they are broken in oxyhaemoglobin, resulting in the liberation of carbon dioxide. Another possibility is, that in oxyhaemoglobin the formation of carbamate is opposed by negatively charged residues. This might be the case for the carbamate formed at the β-subunits where the N-terminal amino group is very near to the C-terminal carboxyl group of the opposite β-subunit.

The third variable which controls the carbon dioxide affinity of haemoglobin is the concentration of *organic phosphate esters* normally found in human erythrocytes. The most important of these is 2,3-diphosphoglycerate (DPG). The crucial significance of this compound for the regulation of the oxygen affinity of haemoglobin inside the erythrocytes will be discussed in the next paragraph. In connection with carbamate formation it is sufficient to mention that the binding of DPG to haemoglobin involves the α-amino groups of the N-termini of the β-subunits (BUNN and BRIEHL, 1970; BENESCH et al., 1971). This binding shifts the ionization

equilibria of these groups towards the protonated form (RIGGS, 1971) and therefore reduces their carbon dioxide affinity.

An antagonism between carbamate formation and binding of DPG was first reported by BAUER (1969) and later verified for a number of experimental conditions (BAUER, 1970; PACE et al., 1970; TOMITA and RIGGS, 1971; SIGGAARD-ANDERSEN, 1971). However, in all of these experiments the change in carbamate affinity was inferred indirectly measuring, for example, the Bohr effect or total carbon dioxide content. It is most significant therefore, that MORROW et al. (1973) observed carbamate formation by nuclear magnetic resonance spectroscopy as ^{13}C resonances and that these typical resonances are very largely inhibited by DPG. This finding confirmed the results obtained by BAUER (1970) on the effect of DPG on the carbon dioxide affinity of human haemoglobin solution and points towards the N-terminal α-amino groups of the β-subunits as the main binding sites for carbon dioxide.

III. Changes of the Oxygen Affinity of Haemoglobin

A. Factors Affecting the Oxygen Affinity of Haemoglobin

A convenient measure of the affinity of haemoglobin for oxygen is the oxygen pressure at which haemoglobin is half saturated with the ligand (half saturation pressure, $P_{\frac{1}{2}}$ or P_{50}). It can be easily obtained from an oxygen haemoglobin equilibrium curve and is inversely related to the oxygen affinity: the higher the oxygen pressure necessary to attain half saturation the lower the oxygen affinity, and vice versa. The number of factors which control the oxygen affinity of haemoglobin is quite large (RIGGS, 1965; ANTONINI, 1965; RIEGEL, 1970; ANTONINI and BRUNORI, 1971) but for most physiological and pathophysiological conditions there are only three variables to consider: 1. temperature, 2. the activity of protons and 3. the concentration of organic phosphate esters.

1. Temperature

Since the reaction of haemoglobin with oxygen is exothermic in nature ($\Delta H = -11$ Kcal/mole) the oxygen affinity of haemoglobin decreases with increasing temperature, i.e., the higher the temperature the higher P_{50}. As pointed out by WYMAN (1948) the heat of reaction of haemoglobin and oxygen is composed of the intrinsic heat of oxygenation, the heat of solution of oxygen and the heat of ionization of the oxygen-linked acid groups. The temperature coefficient for this relationship ($\Delta \log P_{50}/\Delta T$) is between 0.019 (DILL and FORBES, 1941) and 0.024 (ASTRUP et al., 1965). This decrease of oxygen affinity with increasing temperature may be of physiological significance during heavy muscular exercise when the temperature in the muscles increases to about 39° C (SALTIN et al., 1968), causing

an additional liberation of 1–2 Vol. per cent of oxygen from the blood at constant oxygen pressure.

2. *Protons*

In 1904 BOHR et al. discovered that carbon dioxide considerably decreases the oxygen affinity of haemoglobin. BARCROFT (1928) attributed this effect (Bohr effect) entirely to an increase of the proton concentration rather than to a specific effect of carbon dioxide. However, later it was unequivocally demonstrated that, apart from changing pH, carbon dioxide specifically decreases the oxygen affinity of haemoglobin (MARGARIA and GREEN, 1933; HERMANN et al., 1939; KREUZER et al., 1972). The fact that the oxygen affinity of haemoglobin decreases *at constant pH* with increasing pressure of carbon dioxide already furnishes proof that the carbamate reaction is truly oxygen linked, as proposed by FERGUSON and ROUGHTON (1934a, b). The thermodynamic relationship between the effect of oxygen on the binding of carbon dioxide and the effect of carbon dioxide on the oxygen affinity of haemoglobin was deduced in 1923 by ADAIR in a very early example of the "principle of linked function" in haemoglobin which was later so successfully applied by WYMAN (1948, 1963, 1967).

As far as the change of P_{50} with pH is concerned, it was noted that the decrease of oxygen affinity with decreasing pH only holds down to pH 6.0–6.5. At more acid pH values the oxygen affinity increases again (FERRY and GREEN, 1929; GERMAN and WYMAN, 1937). This phenomenon is called the acid Bohr effect in contrast to the alkaline Bohr effect. In any event, from what was said about the negative interaction of oxygen and carbon dioxide binding, one would expect that, in the range where oxygen affinity decreases with increasing acidity (alkaline Bohr effect) the uptake of oxygen by unliganded haemoglobin would lead to a liberation of protons, whereas, in the range of the acid Bohr effect, protons would be taken up by haemoglobin on ligand binding. This concept was experimentally verified by HASTINGS et al. (1924) and GERMAN and WYMAN (1937), and later confirmed by many other workers. The thermodynamic relationship between oxygen affinity and proton activity on the one hand, and proton dissociation and oxygen binding on the other, is as follows (WYMAN, 1948):

$$\left|\frac{\log pO_2}{pH}\right|_Y = \left|\frac{\Delta H^+}{\Delta Y}\right|_{pO_2} \tag{4}$$

and means that the change in oxygen pressure for a given change in pH, at constant oxygen saturation (Y), is numerically equivalent to the number of protons liberated from haemoglobin upon ligand binding. It further implies that the liberation of protons is linearly related to oxygen binding, at least if protons and oxygen are the only interacting partners.

The numerical equivalence of both terms in Equation (4) was experimentally confirmed by ANTONINI et al. (1963) for haemoglobin solutions in the absence of chemicals other than buffers. The presence of carbon dioxide and DPG, however, makes the situation more complex, causing non-identity of the two terms (SIGGAARD-ANDERSEN et al., 1972). Similarly, the linear relationship between ligand

binding and proton release, which was confirmed in a number of experiments in different laboratories (ANTONINI et al., 1965a; GRAY, 1970; OLSON and GIBSON, 1973a), appears to deviate from linearity in the presence of carbon dioxide and DPG (SIGGAARD-ANDERSEN et al., 1972; GARBY et al., 1972). These chemicals are, however, known to have an influence on the Bohr effect by itself so that it is difficult to judge whether the observed deviation is real or due to side reactions of carbon dioxide and DPG with haemoglobin.

The *chemical groups involved in the Bohr effect* (oxygen-linked acid groups) were long suspected to be imidazole (WYMAN, 1948; BENESCH and BENESCH, 1961; ANTONINI et al., 1965b) and α-amino groups (HILL and DAVIS, 1967) in the range of the alkaline Bohr effect, and carboxyl groups (WYMAN, 1948; ANTONINI et al., 1965b) in the range of the acid one. With regard to the alkaline Bohr effect, the studies of KILMARTIN and ROSSI-BERNARDI (1969) and of KILMARTIN and WOOTTON (1970) have brought foreward strong evidence that 25 per cent of the released protons come from the α-amino groups of Val NA (1) of both α-subunits and 50 per cent from the imidazole groups of His HC 3 (146) of the β-subunits. The pK of this latter group has been measured by nuclear magnetic resonance spectroscopy at 30° C and found to be 8.0 in deoxyhaemoglobin and 7.1 in carbonmonoxyhaemoglobin (KILMARTIN et al., 1973). The release of protons upon ligand binding can be explained by specific salt-bridges in deoxyhaemoglobin, one pair between the α-amino groups of Val NA 1 (1) and the carboxyl groups of Arg HC 3 (143) of the α-subunits, and the other between the imidazole group of His HC 3 (146) and the carboxyl group of Asp FG 1 (94) of the β-subunits (PERUTZ et al., 1969; PERUTZ, 1970). These salt-bridges break on oxygen binding, and protons are released.

We note that at least two of the pairs of salt-bridges responsible for the alkaline Bohr effect are also involved in haem-haem interactions. This would lead to the conclusion that the Bohr effect and cooperativity depend on each other. However, this does not seem to be the case. For example, artificial intermediates, i.e., haemoglobin with only either the α-subunits or the β-subunits free to react with ligands, show a Bohr effect almost identical to that of normal haemoglobin, yet cooperativity is greatly reduced (BANERJEE and CASSOLY, 1969; BRUNORI et al., 1970). Furthermore, the extensive studies of BUNN and GUIDOTTI (1972) on the oxygen affinity of normal haemoglobin, measured in a wide range of solvent conditions, point in the same direction, namely that Bohr effect and cooperativity are largely independent. This fact puts a restriction on the validity of the stereochemical model of PERUTZ (1970), because the two allosteric phenomena obviously cannot be based on the same salt-bridges seen by X-ray crystallography. It is much more likely that there are multiple equilibria involved in allostery which have yet to be defined in order to allow a physically meaningful interpretation of allosteric effects (WEBER, 1972).

Physiological Significance of the Bohr Effect. As a decrease of pH leads to a diminution of the oxygen affinity of haemoglobin one would expect the unloading of oxygen from blood in the tissue capillaries to improve as carbon dioxide and fixed acids enter the blood stream. Unfortunately, there is no direct experimental evidence to prove this claim but certain deductions can be made from known changes of pH and pO_2 which occur under certain physiological conditions.

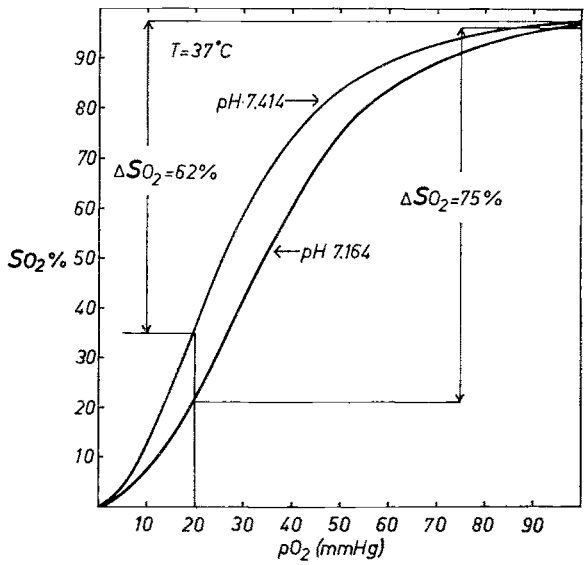

Fig. 4. Effect of a decrease in pH on oxygen haemoglobin equilibrium curves under in vivo conditions. (Based on the data of DOLL et al., 1968)

The magnitude of the Bohr coefficient $\Delta \log P_{50}/\Delta$ pH is about -0.48 (DILL et al., 1940) when changes of pH are brought about by changes of pCO_2, but are lower when the pCO_2 is kept constant and pH is changed by adding either hydrogen or hydoxyl ions (SIGGAARD-ANDERSEN et al., 1972; WRANNE et al., 1972). However, this effect does not need to be taken into account for the following considerations, because *in vivo* the pH is mainly determined by pCO_2 so that the use of a Bohr coefficient of -0.48 is justified. There are two physiological situations in which pH changes are large enough to produce a significant change in oxygen affinity of haemoglobin. Firstly, during the placental gas transfer, and secondly during heavy muscular exercise. In the placenta the maternal and foetal blood stream meet and, as a result of the exchange processes, the maternal blood becomes more acid and the foetal blood more alkaline. Hence, one has a doubling of the Bohr effect. In the maternal blood oxygen is released more readily, since the oxygen affinity decreases, and the foetal blood takes up oxygen more easily because of its increase in oxygen affinity (BARTELS, 1959). Quantitative consideration of this process revealed, however, that the Bohr effect accounts only for about 8 per cent of the oxygen exchanged across the placenta (HILL et al., 1973).

During a submaximal exercise of about 200 watts, the situation is somewhat different. The pO_2 in the venous blood of working muscle is about 20 mm Hg and the pH averages 7.164 under these conditions (DOLL et al., 1968). From the oxygen haemoglobin equilibrium curve (Fig. 4) and from the published values for the increase in blood flow from 2 ml of blood/100 ml of tissue at rest (DE MARÉES and BARBEY, 1973) to about 300 ml of blood/100 ml of tissue at this work load (VARNAUSKAS et al., 1970) it can be estimated that the Bohr effect provides about 20 per cent of the amount of oxygen made available to the working muscle. The

increase of temperature (see above) contributes another 10 per cent, so that the overall effect of changes in oxygen affinity due to a rise in proton concentration and in temperature amounts to 30 per cent of the oxygen delivered to the working muscle.

3. Organic Phosphate Esters

Perhaps the most significant discovery with regard to the regulation of oxygen affinity was the finding that red cell metabolism and haemoglobin function are connected via organic phosphate esters of the red cell, of which DPG and adenosine triphosphate are the most important (BENESCH and BENESCH, 1967; CHANUTIN and CURNISH, 1967; BENESCH et al., 1968b). Particularly DPG, which occurs with haemoglobin in about equimolar concentrations (5 mM/l red blood cells) in human erythrocytes (RAPOPORT and GUEST, 1941) was shown to decrease the oxygen affinity of haemoglobin most effectively. This compound is synthesized in the erythrocytes from 1,3-diphosphoglycerate by the enzyme diphosphoglycerate mutase. The breakdown of DPG occurs via a specific phosphatase (DPG phosphatase) yielding 3-phosphoglycerate and inorganic phosphate. (For a very detailed discussion of the red cell metabolism, and in particular of the DPG shunt, the reader is referred to some recent summarizing articles: RAPOPORT, 1969; RAPOPORT et al., 1972; DUHM, 1973; RØRTH, 1973.)

From the pronounced effect of DPG on the oxygen affinity of haemoglobin it can be deduced that, in analogy to the effect of protons and carbon dioxide, the deoxy conformation of the molecule must have a much higher affinity for the phosphate compound than has the oxy conformation. It has actually been shown by direct binding experiments in dilute haemoglobin solutions that DPG (BENESCH et al., 1968b) and ATP (LO and SCHIMMEL, 1969) combine with deoxyhaemoglobin in a ratio of one mole per haemoglobin tetramer. This binding is specific for the deoxy conformation, since binding to oxyhaemoglobin is negligible under these conditions (BENESCH et al., 1968b; LO and SCHIMMEL, 1969; CALDWELL et al., 1971; BAUER et al., 1973).

The deoxy conformation is therefore stabilized by DPG (as it is by protons and carbon dioxide) and becomes more resistant to oxygenation by a change in free energy of -6.5 Kcal/mole which accompanies the complex formation of DPG with haemoglobin (BENESCH et al., 1969). At the high haemoglobin concentration in the red blood cell the situation appears to be more complex, as indicated by the results of GARBY and DE VERDIER (1971) who found the binding of DPG to decrease with increasing haemoglobin concentration, and by the data of KLINGER et al. (1971) who were able to separate three classes of binding of ATP to oxyhaemoglobin, one of them being very firm.

The *binding site of DPG* is associated with the β-subunits (BENESCH et al., 1968a) where DPG is bound at the entrance to the central cavity which runs through the molecule along the dyad axis of the tetramer. At this particular place there are located a number of positively charged residues which can interact electrostatically with the polyanion that carries about four negative charges at neutral pH (BENESCH et al., 1969). From the lesser effect of DPG on the oxygen affinity of foetal

haemoglobin in comparison to HbA (BAUER et al., 1968, 1969; TYUMA and SHIMIZU, 1969) it was deduced that one residue involved in the binding of DPG is the imidazole group of histidine H 21 (143) (DE VERDIER and GARBY, 1969). This is located at the entrance to the central cavity and is replaced in the γ-subunits of foetal haemoglobin by a neutral serine which evidently reduces the electrostatic interactions with the polyanion.

Affinity labelling with pyridoxal phosphate (RENTHAL et al., 1970; BENESCH et al., 1972) and studies on the reactivity of acetylated foetal haemoglobin towards DPG (BUNN and BRIEHL, 1970), as well as the depression of the binding of carbon dioxide by DPG (BAUER, 1969, 1970), traced the α-amino group of Valine NA 1 (1) of the β-subunits as another residue involved in the binding of the phosphate compound. All the conclusions concerning the stoicheometry of binding and the binding sites themselves were confirmed by the X-ray diffraction studies of ARNONE (1972). ARNONE showed that there is only one molecule of DPG bound per molecule of deoxyhaemoglobin and that, apart from the basic groups of Valine NA 1 (1) β_1, β_2 and Histidine H 21 (143) β_1, β_2, the imidazole group of His NA 2 (2) and the ε-amino group of lysine EF 6 (82) of the β-subunits also interact with DPG. The place where the polyanion is plugged between the two β-subunits is thus surrounded with a wreath of positive charges which firmly anchor the molecule in the deoxy conformation of haemoglobin. In fully liganded haemoglobin, on the other hand, the room between the β-subunits becomes much more narrow and extended so that DPG can no longer be accommodated.

One interesting aspect of the binding of DPG concerns the Bohr effect. The binding of DPG would tend to increase the pK values of the six cationic groups (4 imidazole and two α-amino groups), whereas the pK of the phosphate groups of DPG would decrease. From the studies on the effect of DPG on the Bohr effect one must conclude that the increase in pK of the cationic groups overcomes the decrease in pK of the phosphate groups because there are more protons liberated upon ligand binding in the presence of DPG than in its absence (BAILEY et al., 1970; DE BRUIN et al., 1971; RIGGS, 1971; DE BRUIN and JANSSEN, 1973). Therefore, in addition to the protons coming from the oxygen-linked acid groups, there are protons released from the groups to which DPG is bound. This explains the enhancement of the Bohr effect by DPG.

Probably the most important *physiological role of DPG* is to reduce the oxygen affinity of haemoglobin in such a way that significant unloading of oxygen is guaranteed at the oxygen pressures occuring in the human body, i.e., to shift the oxygen haemoglobin equilibrium curve to the position where it is actually found under normal conditions. From Fig. 5 it can be seen that the removal of organic phosphates from human erythrocytes increases the oxygen affinity of haemoglobin to such an extent that the amount of oxygen, which can be removed from the blood, decreases by more than 60 per cent.

It might be argued that such a marked increase of the oxygen affinity does not have these adverse effects on tissue oxygenation because the carriers of haemoglobin Yakima who have about the same oxygen affinity, do not show any clinical signs except a high haemoglobin concentration (NOVY et al., 1967; METCALFE and DHINDSA, 1972). However, the combination of a high haematocrit and a high oxygen affinity does not seem to be the optimal solution for systemic oxygen

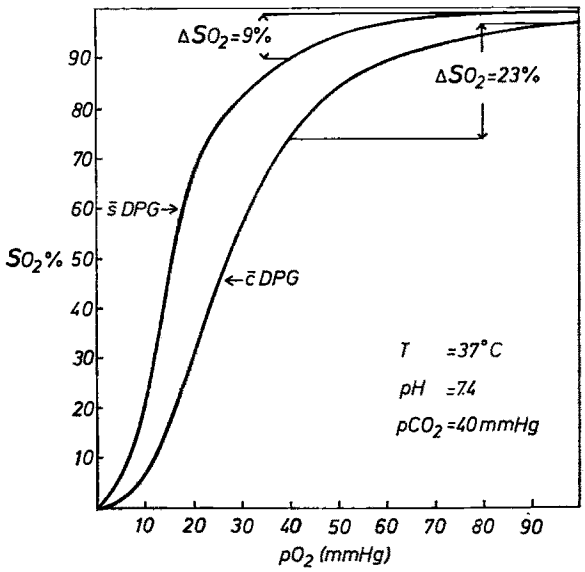

Fig. 5. Effect of removal of DPG from human erythrocytes on the amount of oxygen liberated from haemoglobin. (Based on the data of DUHM, 1971)

transport because of the increased viscosity of the blood under these conditions (RICHARDSON and GUYTON, 1959).

B. Physiological and Pathophysiological Implications of Changes in Oxygen Affinity of Haemoglobin

So far we have discussed the physiological implications of a change in the oxygen affinity only in connection with single parameters which have an influence on the reaction of haemoglobin with oxygen. In the following paragraph an attempt will be made to consider oxygen affinity in connection with other parameters governing oxygen delivery to the tissues, e.g., the oxygen capacity of the blood and cardiac output.

The physiological advantages of a decrease in oxygen affinity of haemoglobin are usually assessed theoretically by showing that, at a given pressure of oxygen in the arterial and venous blood, the amount of oxygen which may become available to the tissues increases with a decrease in oxygen affinity (see Figs. 4 and 5). This is, however, only true as long as the arterial oxygen pressure is high enough to keep the arterial oxygen saturation in the upper flat part of the oxygen haemoglobin equilibrium curve. At high altitude, for example, where the oxygen pressure of the arterial blood is greatly reduced, a very low oxygen affinity is not beneficial (KEYS et al., 1936; BAUMANN et al., 1971; BANCHERO and GROVER, 1972) and may significantly jeopardize oxygen uptake in the lungs.

The circumstantial evidence for the favourable effect of *adaptations of the oxygen affinity* is extensive and comprises studies in comparative biology (BARTELS, 1964; METCALFE and DHINDSA, 1972; JOHANSEN and LENFANT, 1972), high altitude (KEYS et al., 1936; ASTE-SALAZAR and HURTADO, 1944; RØRTH et al., 1972; LENFANT et al., 1968, 1971; HARTLEY, 1972) and various clinical conditions usually associated with what is periphrastically called "tissue hypoxia", e.g., red cell mass deficit and cardiopulmonary insufficiency. In order to *quantitively* assess the effect of changes in oxygen affinity of haemoglobin on the oxygen delivery to the tissues, one would like to have a precise measure of the state of oxygenation in the tissues. Basically there are two methods available to obtain information on this parameter under physiological conditions. One is to measure pO_2 directly with microelectrodes (LÜBBERS et al., 1971) and the other is to use myoglobin as an indicator for the state of oxygenation in the skeletal and cardiac muscle (COBURN and MAYERS, 1971; COBURN et al., 1973). A very informative approach to this problem has been used by VERSMOLD and BRAUSER (1973) who employed the spectra of cytochrome a in a perfused liver preparation to demonstrate a marked shift of oxygen from haemoglobin to the tissues upon addition of DPG. All of these methods do require, however, a fair amount of instrumentation and are as yet restricted to a few specialised laboratories.

To circumvent these problems some investigators have choosen to measure oxygen consumption, cardiac output and the oxygen content and pressure in the arterial and mixed venous blood in order to find out the effect of oxygen affinity changes on systemic oxygen transport. From the few studies in which these variables have been measured it can be inferred that, *at rest*, variations in the oxygen affinity do not lead to variations in the cardiac output but rather to changes of the haemoglobin concentration, i.e., the oxygen carrying capacity of blood. Thus, it has been shown that the arterio-venous oxygen concentration difference (AVD_{O_2}) and the mixed venous pO_2 are quite similar in sheep and human subjects in spite of the fact that the oxygen affinity of sheep blood is very much lower than that of human blood. This close similarity in AVD_{O_2} is due to the low oxygen capacity of sheep in comparison with human blood. Since the oxygen consumption is almost identical in both species, it follows that the cardiac output must also be very similar which is indeed the case (PARER et al., 1967). The relationship between oxygen affinity and oxygen capacity, found in comparing two different species, is likewise observed during the postnatal development within the same species. BARTELS and colleagues (1963) have studied systemic oxygen transport in lambs and kids after birth and found that in spite of a marked decrease in oxygen affinity due to the replacement of foetal by adult haemoglobin, AVD_{O_2} and mixed venous oxygen pressure remained essentially unaltered as a result of the decrease in oxygen capacity. A third example is found in human subjects who are heterozygous for haemoglobin Yakima, a haemoglobin mutant which leads to a high oxygen affinity (NOVY et al., 1967). This abnormality in the respiratory function of the blood is compensated for by an increase in oxygen capacity of the blood, with the result that AVD_{O_2} and mixed venous pO_2 are within the normal limits, as are cardiac output and oxygen consumption.

From these examples it is clear that, in a number of instances, the oxygen affinity of blood is one of the regulating factors for erythropoiesis. The regulatory

mechanism is probably brought about by the fact that renal blood flow and renal arterio-venous oxygen concentration difference are quite insensitive to variations in cardiac output and arterial oxygen pressure. Due to the low renal AVD_{O_2} the blood enters and leaves the kidneys mostly along the upper, flat part of the oxygen haemoglobin equilibrium curve, where slight variations in oxygen saturation lead to large variations in the oxygen pressure in the venous blood leaving the kidneys. It is probably this latter parameter which transmits changes of the oxygen affinity to the erythropoietin-producing system, so that both oxygen capacity and oxygen affinity act together to secure an optimal oxygen pressure in the tissue capillaries (ADAMSON et al., 1969; PARER, 1970; VERSMOLD et al., 1972; METCALFE and DHINDSA, 1972).

Many *clinical situations* in which changes of the oxygen affinity may be of some importance are characterized as hypoxic conditions. This indicates that the oxygen concentration in the arterial blood is below the normal value of about 20 ml/100 ml of blood either due to a deficiency in red cell mass (anaemic hypoxia) or due to a reduced oxygen pressure in the arterial blood as seen at high altitude or associated with disorders of lung and heart (hypoxic hypoxia). These hypoxic conditions induce a decrease of the oxygen affinity of blood which is thought to partly compensate for the reduced concentration of oxygen in the arterial blood. The decrease of the oxygen affinity is brought about by a concomitant rise of the DPG concentration in the erythrocytes, where the phosphate ester exerts its effect not only by direct interaction with haemoglobin but also via changes of the intra-erythrocytic pH value (BATTAGLIA et al., 1970; BELLINGHAM et al., 1971; DUHM, 1971).

The *regulation of synthesis and breakdown of DPG under these hypoxic conditions* has been a matter of some controversy but it is now well established that the pH value inside the erythrocytes is the most important determinant of the level of DPG. An increase of pH leads, in general, to an increase in the concentration of DPG, whereas a rise of acidity causes DPG to fall. Variations in the degree of oxygenation of haemoglobin are accompanied by changes of the intraerythrocytic pH in such a manner that a decrease of the oxygen saturation leads to an increase in pH which, in turn, enhances the synthesis of DPG and cuts down its degradation (ASAKURA et al., 1966; RØRTH, 1970; DUHM and GERLACH, 1971; LENFANT et al., 1971; RØRTH et al., 1972; MOORE et al., 1972). This increase in red cell pH upon deoxygenation is caused by the lower net negative charge of deoxyhaemoglobin in comparison to oxyhaemoglobin which leads to a redistribution of diffusable charges according to the DONNAN equilibrium (VAN SLYKE et al., 1925). Any significant increase of the concentration of deoxyhaemoglobin is therefore transmitted to the system which controls the respiratory properties of blood almost exclusively via an increase in pH (RØRTH, 1970; DUHM and GERLACH, 1971).

This brilliant example of biological regulation in which a characteristic feature of haemoglobin is used to monitor and control its function with the aid of red cell metabolism operates in a number of hypoxic conditions. At high altitude for example a decrease in oxygen affinity is parallelled, or rather caused, by an increased concentration of DPG, as was first shown by LENFANT and his colleagues (1968) and later amply verified not only for man (LENFANT et al., 1971; RØRTH

et al., 1972) but also for rats and guinea pigs (BAUMANN et al., 1971; DUHM and GERLACH, 1971). Similar changes are observed when the arterial oxygen content is reduced in anaemia (EATON and BREWER, 1968; VALERI and FORTIER, 1969; DUHM and GERLACH, 1971; VALERI et al., 1972) and in patients with cardiopulmonary insufficiency (OSKI et al., 1969; VALERI and FORTIER, 1969; WOODSON et al., 1970; VALERI et al., 1972). In all of these cases the average concentration of deoxyhaemoglobin is higher than it is under normal conditions; as a result the average pH value in the erythrocytes increases. This small rise in pH causes the concentration of DPG to augment and the affinity of haemoglobin for oxygen to decrease.

Although the relationship between hypoxaemia and the decrease in oxygen affinity is well documented, little is known about the *quantitative* contribution of this effect to systemic oxygen transport. First of all it must be remembered that the oxygen affinity of blood is usually assessed under standard conditions in respect to pH and partial pressure of carbon dioxide. Thus, the oxygen affinity *in vivo* and *in vitro* may differ quite considerably as is seen during the first period of adaptation to high altitude where the decrease of oxygen affinity due to accumulation of DPG is offset by the increase in pH due to hyperventilation (RØRTH et al., 1972). In view of this it seems that discussions on the effect of changes of the oxygen affinity on the uptake or delivery of oxygen by the blood, should be based as much as possible on parameters measured in vivo. One example of the beneficial effect of a decreased oxygen affinity upon exposure to an altitude of 4350 meters above sea level is furnished by a study in which the oxygen uptake was measured at different work loads and after different periods of time of exposure (HARTLEY, 1972). While maximal oxygen uptake at high altitude was lower than at sea level, it increased from the value after 1 hour of exposure (when oxygen affinity was yet unchanged) by about 20 per cent after two or ten days at altitude (when oxygen affinity had decreased). This rise in maximal oxygen uptake was associated with a corresponding decrease in oxygen content of the mixed venous blood which coincided with the period of decreased oxygen affinity of blood. A similar effect was described in two exercising patients who had the same degree of anaemia but with widely different oxygen affinities due to inherited enzyme defects of the red cell. The patient with the low oxygen affinity exhibited a much larger fall in the oxygen saturation of the mixed venous blood than did the patient having the high oxygen affinity, in spite of the fact that the latter had a more pronounced fall of the mixed venous oxygen pressure. This diminished oxygen extraction resulted in a compensatory increase of cardiac output (DELIVORIA-PAPADOPOULOS et al., 1969; OSKI et al., 1971).

In summarizing these considerations on the interrelationship between changes in oxygen affinity and systemic oxygen transport, the following conclusions can be made. Whenever the erythroid generating tissue is functioning, the oxygen affinity appears to play an important role as regulator of erythropoiesis. Since it is the "tissue" pressure of oxygen which determines erythropoietic activity (THORLING and ERSLEV, 1968) changes of the oxygen affinity may well be reflected in the oxygen pressure at the sites which control erythropoietic activity. In favour of such an interpretation are recent results of VERSMOLD et al. (1972) and MILLER et al. (1973) who demonstrated a decrease of erythropoietic activity under conditions of decreased oxygen affinity. The same reasoning can be applied to the study of BELLINGHAM and HUEHNS (1968), who compared patients with haemolytic disease

caused by abnormal haemoglobins. The patients with a normal or reduced oxygen affinity had a lower red cell mass than had the patients with an increased oxygen affinity. It therefore appears that one mechanism by which a change of the oxygen affinity influences the state of oxygenation of the tissues is via the regulation of the haemoglobin concentration, i.e., the oxygen carrying capacity of the blood.

A *direct improvement* of the flow of oxygen from the blood to the tissues by a decreased oxygen affinity is certainly important under extreme conditions when the need of the organism for oxygen is greatly increased as, for example, during muscular exercise in hypoxic conditions. Under these circumstances the favourable effect of a decreased oxygen affinity can be seen directly in terms of an increase of maximal oxygen uptake or an increased economy of cardiac performance.

IV. Physiological Aspects of the Transport of Carbon Dioxide in the Blood

Carbon dioxide is transported in the blood as bicarbonate, carbamate and in the dissolved form. The hydration reaction of carbon dioxide which takes place in the blood yields bicarbonate ions and protons and is accelerated to the necessary extent in the red cells by the enzyme carbonic anhydrase. In the lungs a dehydration reaction takes place that leads to the formation of molecular carbon dioxide. Besides the enzyme carbonic anhydrase, it is the linkage between oxygen binding to haemoglobin on the one hand, and the liberation of hydrogen ions and carbon dioxide on the other which is of crucial importance for the understanding of carbon dioxide transport in the blood.

The reduction of carbon dioxide content in blood on oxygenation at constant pCO_2 was conclusively demonstrated by CHRISTIANSEN et al. in 1914. Whilst the existence of this phenomenon is universally accepted, there is still disagreement on the relative importance of the chemical mechanisms involved in the CHRISTIANSEN-DOUGLAS-HALDANE effect (CDH effect), as it is commonly known. CHRISTIANSEN and her colleagues, and later WYMAN (1948), discussed the possibility that oxyhaemoglobin is a stronger acid than deoxyhaemoglobin is, and that, therefore, the diminution of carbon dioxide binding could be almost fully explained by the lesser bicarbonate content in oxygenated blood due to the fall in pH.

ROUGHTON (1964), on the other hand, concluded that about 70 per cent of the CDH effect can be explained by the decrease in carbamate binding to haemoglobin when the protein combines with oxygen. It is, however, well established now that DPG binding to haemoglobin and carbamate formation are antagonistic processes (BAUER, 1969, 1970; PACE et al., 1970; SIGGAARD-ANDERSEN, 1971; CALDWELL et al., 1971; BRENNA et al., 1972; WRANNE et al., 1972; MORROW et al., 1973) and that, therefore, the role of carbamino compounds in the CDH effect of human blood can only be assessed under conditions which permit careful control of the concentration of organic phosphate compounds in red blood cells. If this is done,

it can easily be calculated from the data of BAUER and SCHRÖDER (1972) that under physiological conditions (pH 7.4, $pCO_2 = 40$ mm Hg) the fraction of oxylabile carbamate is 0.092 Moles CO_2/Mole O_2 and that therefore the contribution of carbamate to the CDH effect, which amounts to 0.28 Moles CO_2/Mole O_2 (KEYS et al., 1936; HARMS and BARTELS, 1961), is only about 33 per cent, compared to the 70 per cent in the earlier estimates (ROUGHTON, 1964).

What are the implications of the reduced carbamate formation for the overall exchange of carbon dioxide during the respiratory cycle? In order to draw any conclusions in this respect one has to remember that the CDH effect is composed of two mechanisms: one is the liberation of protons as oxygen is bound, leading to a reduction of the *bicarbonate concentration* in oxygenated blood. The other is the difference in *carbamate concentration* between oxygenated and deoxygenated blood. From the reaction scheme of the carbamate formation it can be seen that a decrease of carbamate formation leads to an absorption of protons. Therefore, the reduction of carbamate formation in the process of oxygenation is coupled with an uptake of protons. At the same time there are protons released by the oxygen-linked acid groups and, as a result of these two opposing processes, the number of protons actually liberated from haemoglobin upon oxygenation are lower in the presence of carbon dioxide than in its absence (ROSSI-BERNARDI and ROUGHTON, 1967; SIGGAARD-ANDERSEN, 1971). Therefore, one would expect that a reduction in carbamate formation, e.g. by DPG, increases the number of protons liberated from haemoglobin upon oxygenation, as is indeed seen to be the case (BAUER, 1970; SIGGAARD-ANDERSEN, 1971; SIGGAARD-ANDERSEN and SALLING, 1971).

The consequences of these interactions on the CDH effect are clear. The decrease of the contribution of oxylabile carbamate is compensated for, at least to a significant extent, by the increase in the number of protons being liberated from haemoglobin. In harmony with this conclusion are the results of KLOCKE (1973) who has measured both the CDH effect and the fraction of oxylabile carbamate as a function of the concentration of DPG. From his data it can be seen that the fraction of oxylabile carbamate decreases by about 60 per cent when the concentration of DPG is increased from zero to normal at pH 7.4, but that the CDH effect is diminished by only about 20 per cent.

In order to assess the *physiological significance of the CDH effect*, one has to remember that, with a normal respiratory exchange ratio of 0.85, 0.85 moles of carbon dioxide are given up in the lungs per mole of oxygen uptake. Oxylabile carbon dioxide exchange (CDH effect), on the other hand, results in the release of 0.28 moles carbon dioxide per mole of oxygen bound. Therefore, the proportion of total exchange of carbon dioxide which is linked to oxylabile reactions is $0.28/0.85 = 33$ per cent. About one third of this latter number is due to oxylabile carbamate, whilst the remaining two thirds can be attributed to the change in acidity of haemoglobin on its combination with oxygen. It follows that it is only the mechanism of the CDH effect which needs revision with regard to former conceptions (ROUGHTON, 1964; ROSSI-BERNARDI and ROUGHTON, 1967), but not its physiological significance. Thus, the interaction between haemoglobin and its ligands in the transport of carbon dioxide provides another illustrative example of the physiological effectiveness of the molecule.

References

ADAIR, G. S.: Thermodynamical proof of the reciprocal relationship of oxygen and carbon dioxide in blood. J. Physiol. (Lond.) **58**, IV–VP (1923).

ADAMSON, J. W., PARER, J. T., STAMATOYANNOPOULOS, G.: Erythrocytosis associated with hemoglobin Rainier: Oxygen equilibria and marrow regulation. J. clin. Invest. **48**, 1376–1386 (1969).

ANTONINI, E.: Interrelationship between structure and function in hemoglobin and myoglobin. Physiol. Rev. **45**, 123–170 (1965).

ANTONINI, E., BRUNORI, M.: Hemoglobin and myoglobin in their reactions with ligands. Amsterdam-London: North-Holland Publ. Comp. 1971.

ANTONINI, E., SCHUSTER, T. M., BRUNORI, M., WYMAN, J.: The kinetics of the Bohr effect in the reaction of human hemoglobin with carbon monoxide. J. biol. Chem. **240**, PC 2262–PC 2264 (1965a).

ANTONINI, E., WYMAN, J., BRUNORI, M., BUCCI, E., FRONTICELLI, C., ROSSI-FANELLI, A.: Studies on the relations between molecular and functional properties of hemoglobin. IV. The Bohr effect in human hemoglobin measured by proton binding. J. biol. Chem. **238**, 2950–2957 (1963).

ANTONINI, E., WYMAN, J., BRUNORI, M., FRONTICELLI, C., BUCCI, E., ROSSI-FANELLI, A.: Studies on the relations between molecular and functional properties of hemoglobin. V. The influence of temperature on the Bohr effect in human and horse hemoglobin. J. biol. Chem. **240**, 1096–1103 (1965b).

ARNONE, A.: X-ray diffraction study of binding of 2,3-diphosphoglycerate to human deoxyhaemoglobin. Nature (Lond.) **237**, 146–149 (1972).

ASAKURA, T., SATO, S., MINAKAMI, S., YOSHIKAWA, H.: Effect of deoxygenation of intracellular hemoglobin on red cell glycolysis. J. Biochem. **59**, 524–526 (1966).

ASTE-SALAZAR, H., HURTADO, A.: The affinity of hemoglobin for oxygen at sea level and at high altitudes. Amer. J. Physiol. **142**, 733–743 (1944).

ASTRUP, P., ENGEL, K., SEVERINGHAUS, J. W., MUNSON, E.: The influence of temperature and pH on the dissociation curve of oxyhemoglobin of human blood. Scand. J. clin. Lab. Invest. **17**, 515–523 (1965).

BAILEY, J. E., BEETLESTONE, J. G., IRVINE, D. H.: Reactivity differences between haemoglobins. Part XVII. The variability of the Bohr effect between species and the effect of 2,3-diphosphoglyceric acid on the Bohr effect. J. chem. Soc. (A) **5**, 756–762 (1970).

BANCHERO, N., GROVER, R. F.: Effect of different levels of simulated altitude on O_2 transport in llama and sheep. Amer. J. Physiol. **222**, 1239–1245 (1972).

BANERJEE, R., ALPERT, Y., LETERRIER, F., WILLIAMS, R. J. P.: Visible absorption and electron spin resonance spectra of the isolated chains of human hemoglobin. Discussion of chain-mediated heme-heme interaction. Biochemistry **8**, 2862–2867 (1969).

BANERJEE, R., CASSOLY, R.: Oxygen equilibria of human hemoglobin valency hybrids. Discussion on the intrinsic properties of α- and β-chains in the native protein. J. Mol. Biol. **42**, 351–361 (1969).

BANERJEE, R., STETZKOWSKI, F., HENRY, Y.: Reciprocal effects of change of subunit structure on ligand equilibria of haemoglobin valency hybrids. Attempted correlation with Electron Paramagnetic Resonance Spectra. J. Mol. Biol. **73**, 455–467 (1973).

BARCROFT, J.: The respiratory function of blood. II. Haemoglobin. Cambridge: Cambridge University Press 1928.

BARTELS, H.: Chemical factors affecting oxygen carriage and transfer from maternal to foetal blood. In: Oxygen supply to the human foetus. Oxford: Blackwell 1959.

BARTELS, H.: Comparative physiology of oxygen transport in mammals. Lancet **19**, 599–604 (1964).

BARTELS, H., HILPERT, P., RIEGEL, K.: Gaswechsel und Herzzeitvolumen während der ersten Lebenswochen. Pflügers Arch. **277**, 61–75 (1963).

BATTAGLIA, F. C., MCGAUGHEY, H., MAKOWSKI, E. L., MESCHIA, G.: Postnatal changes in oxygen affinity of sheep red cells: a dual role of diphosphoglyceric acid. Amer. J. Physiol. **219**, 217–221 (1970).

BAUER, C.: Antagonistic influence of CO_2 and 2,3-diphosphoglycerate on the Bohr effect of human haemoglobin. Life Sci. (II) **8**, 1041–1046 (1969).

BAUER, C.: Reduction of the carbon dioxide affinity of human haemoglobin solutions by 2,3-diphosphoglycerate. Resp. Physiol. **10**, 10–19 (1970).

BAUER, C., HENRY, Y., BANERJEE, R.: Binding of 2,3-diphosphoglycerate to haemoglobin valency hybrids. Nature (New Biol.) **242**, 208–209 (1973).

BAUER, C., LUDWIG, I., LUDWIG, M.: Different effects of 2,3-diphosphoglycerate and adenosine triphosphate on the oxygen affinity of human adult and foetal haemoglobin. Life Sci. (I) **7**, 1339–1343 (1968).

BAUER, C., LUDWIG, M., LUDWIG, I., BARTELS, H.: Factors governing the oxygen affinity of human adult and foetal blood. Resp. Physiol. **7**, 271–277 (1969).

BAUER, C., SCHRÖDER, E.: Carbamino compounds of haemoglobin in human adult and foetal blood. J. Physiol. (Lond.) **227**, 457–471 (1972).

BAUMANN, R., BAUER, C., BARTELS, H.: Influence of chronic and acute hypoxia on oxygen affinity and red cell 2,3-diphosphoglycerate of rats and guinea pigs. Resp. Physiol. **11**, 135–144 (1971).

BELLINGHAM, A. J.: The physiological significance of the Hill parameter "n". Scand. J. Haemat. **9**, 552–556 (1972).

BELLINGHAM, A. J., DETTER, J. C., LENFANT, C.: Regulatory mechanisms of hemoglobin oxygen affinity in acidosis and alkalosis. J. clin. Invest. **50**, 700–706 (1971).

BELLINGHAM, A. J., HUEHNS, E. R.: Compensation in haemolytic anaemias caused by abnormal haemoglobins. Nature (Lond.) **218**, 924–926 (1968).

BENESCH, R., BENESCH, R. E.: The chemistry of the Bohr effect. I. The reaction of N-ethylmaleimide with oxygen linked acid groups of hemoglobin. J. biol. Chem. **236**, 405–410 (1961).

BENESCH, R., BENESCH, R. E.: Some relations between structure and function in hemoglobin. J. Mol. Biol. **6**, 498–505 (1963).

BENESCH, R., BENESCH, R. E.: The effect of organic phosphates from the human erythrocyte on the allosteric properties of hemoglobin. Biochem. Biophys. Res. Commun. **26**, 162–167 (1967).

BENESCH, R., BENESCH, R. E., ENOKI, Y.: The interaction of hemoglobin and its subunits with 2,3-diphosphoglycerate. Proc. nat. Acad. Sci. (Wash.) **59**, 1102–1106 (1968a).

BENESCH, R., BENESCH, R. E., YU, C. I.: Reciprocal binding of oxygen and diphosphoglycerate by human hemoglobin. Proc. nat. Acad. Sci. (Wash.) **59**, 526–532 (1968b).

BENESCH, R. E., BENESCH, R., BANK, A., RENTHAL, R., BRAY, B. A.: The preparation and properties of pyridoxilated hemoglobin. In: Genetical, functional and physical studies of hemoglobin. Basel: Karger 1971.

BENESCH, R. E., BENESCH, R., RENTHAL, R., MAEDA, N.: Affinity labeling of the polyphosphate binding site of hemoglobin. Biochemistry **11**, 3576–3582 (1972).

BENESCH, R. E., BENESCH, R., YU, C. I.: The oxygenation of hemoglobin in the presence of 2,3-diphosphoglycerate. Effect of temperature, pH, ionic strength and hemoglobin concentration. Biochemistry **8**, 2567–2571 (1969).

BENESCH, R. E., RANNEY, H., BENESCH, R., SMITH, G. M.: The chemistry of the Bohr effect. II. Some properties of hemoglobin H. J. biol. Chem. **236**, 2926–2929 (1961).

BOHR, C.: Theoretische Behandlung der quantitativen Verhältnisse bei der Sauerstoffaufnahme des Hämoglobins. Zbl. Physiol. **17**, 682–691 (1903).

BOHR, C., HASSELBALCH, K., KROGH, A.: Über einen in biologischer Beziehung wichtigen Einfluß, den die Kohlensäurespannung des Blutes auf dessen Sauerstoffspannung übt. Scand. Arch. Physiol. **16**, 402–412 (1904).

BOLTON, W., PERUTZ, M. F.: Three dimensional Fourier synthesis of horse deoxyhaemoglobin at 2.8 Å resolution. Nature (Lond.) **228**, 551–552 (1970).

BRAUNITZER, G.: Die Primärstruktur der Eiweißstoffe. Verh. Ges. dtsch. Naturf. u. Ärz. **54**, 407–417 (1967).

BRAUNITZER, G., HILSE, K., RUDLOFF, V., HILSCHMANN, N.: The hemoglobins. Adv. Protein Chem. **19**, 1–71 (1964).

BRENNA, O., LUZZANA, M., PACE, M., PERRELLA, M., ROSSI, F., ROSSI-BERNARDI, L., ROUGHTON, F. J. W.: The interaction between hemoglobin and its oxygen-linked ligands. In: Hemoglobin and red cell structure and function. New York-London: Plenum Press 1972.

BRUIN DE, S. H., JANSSEN, L. H. M.: The interaction of 2,3-diphosphoglycerate with human hemoglobin. Effects on the alkaline and acid Bohr effect. J. biol. Chem. **248**, 2774–2777 (1973).

Bruin de, S. H., Janssen, L. H. M., van Os, G. A. J.: Effect of 2,3-diphosphoglycerate on the Bohr effect of human adult hemoglobin. Biochem. Biophys. Res. Commun. **45**, 544–550 (1971).

Brunori, M., Amiconi, G., Antonini, E., Wyman, J., Winterhalter, K. H.: Artificial intermediates in the reaction of haemoglobin. Functional and conformational properties of the cyanmet intermediates. J. Mol. Biol. **49**, 461–471 (1970).

Bunn, H. F., Briehl, R. W.: The interaction of 2,3-diphosphoglycerate with various human hemoglobins. J. clin. Invest. **49**, 1088–1095 (1970).

Bunn, H. F., Guidotti, G.: Stabilizing interactions in hemoglobin. J. biol. Chem. **247**, 2345–2350 (1972).

Caldwell, P. R. B., Nagel, R. L., Jaffé, E. R.: The effect of oxygen, carbon dioxide, pH and cyanate on the binding of 2,3-diphospholgycerate to human hemoglobin. Biochem. Biophys. Res. Commun. **44**, 1504–1509 (1971).

Cassoly, R., Gibson, Q. H.: The kinetics of ligand binding to hemoglobin valency hybrids and the effect of anions. J. biol. Chem. **247**, 7332–7341 (1972).

Chanutin, A., Curnish, R. R.: Effect of organic and inorganic phosphate on the oxygen equilibrium of human erythrocytes. Arch. Biochem. Biophys. **121**, 96–102 (1967).

Christiansen, J., Douglas, C. G., Haldane, J. S.: The absorption and dissociation of carbon dioxide by human blood. J. Physiol. (Lond.) **48**, 244–271 (1914).

Coburn, R. F., Mayers, L. B.: Myoglobin oxygen tension determined from measurements of carboxymyoglobin in sceletal muscle. Amer. J. Physiol. **220**, 66–74 (1971).

Coburn, R. F., Ploegmakers, F., Gondrie, P., Abboud, R.: Myocardial myoglobin oxygen tension. Amer. J. Physiol. **224**, 870–876 (1973).

Deal, W. J., Mohlman, S. G., Sprang, M. L.: Conformational equilibria in spin-labeled hemoglobin. Science **171**, 1147–1149 (1970).

Delivoria-Papadopoulos, M., Oski, F. A., Gottlieb, A. J.: Oxygen-hemoglobin dissociation curves. Effect of inherited enzyme defects of the red cell. Science **165**, 601–602 (1969).

Dill, D. B., Forbes, W. H.: Respiratory and metabolic effects of hypothermia. Amer. J. Physiol. **132**, 685–697 (1941).

Dill, D. B., Graybiel, A., Hurtado, A., Taquini, A. C.: Der Gasaustausch in der Lunge im Alter. Z. Alternsforsch. **2**, 20–33 and 172 (1940).

Doll, E., Keul, J., Maiwald, C.: Oxygen tension and acid-base equilibria in venous blood of working muscle. Amer. J. Physiol. **215**, 23–29 (1968).

Duhm, J.: Effects of 2,3-diphosphoglycerate and other organic phosphate compounds on oxygen affinity and intracellular pH of human erythrocytes. Pflügers Arch. **326**, 341–356 (1971).

Duhm, J.: 2,3-diphosphoglycerate metabolism of erythrocytes and oxygen transport function. In: Erythrocytes, Thrombocytes, Leucocytes. Stuttgart: Thieme 1973.

Duhm, J., Gerlach, E.: On the mechanism of the hypoxia induced increase of 2,3-diphosphoglycerate in erythrocytes. Pflügers Arch. **326**, 254–269 (1971).

Eaton, J. W., Brewer, G. J.: The relationship between red cell 2,3-diphosphoglycerate and levels of hemoglobin in the human. Proc. nat. Acad. Sci. (Wash.) **61**, 756–760 (1968).

Edelstein, S. J.: Extensions of the allosteric model for haemoglobin. Nature (Lond.) **230**, 224–227 (1971).

Faurhault, C.: Études sur les solutions aqueuses de carbamates et de carbonates. J. Chim. Phys. **22**, 1–44 (1925).

Ferguson, J. K. W.: Carbamino compounds of CO_2 with human haemoglobin and their role in the transport of CO_2. J. Physiol. (Lond.) **88**, 40–55 (1936).

Ferguson, J. K. W., Roughton, F. J. W.: The direct chemical estimation of carbamino compounds of CO_2 with haemoglobin. J. Physiol. (Lond.) **83**, 68–86 (1934a).

Ferguson, J. K. W., Roughton, F. J. W.: The chemical relationships and physiological importance of carbamino compounds of CO_2 with haemoglobin. J. Physiol. (Lond.) **83**, 87–102 (1934b).

Ferry, R. M., Green, A. A.: Studies in the chemistry of hemoglobin. III. The equilibrium between oxygen and hemoglobin and its relation to changing hydrogen ion activity. J. biol. Chem. **81**, 175–203 (1929).

Forster, R. E., Constantine, H. P., Craw, M. R., Rotman, H. H., Klocke, R. A.: Reaction of CO_2 with human hemoglobin solution. J. biol. Chem. **243**, 3317–3326 (1968).

GARBY, L., DE VERDIER, D. H.: Affinity of human hemoglobin A to 2,3-diphosphoglycerate. Effect of hemoglobin concentration and of pH. Scand. J. clin. Lab. Invest. **27**, 345–350 (1971).

GARBY, L., ROBERT, M., ZAAR, B.: Proton- and carbamino-linked oxygen affinity of normal human blood. Acta Physiol. Scand. **84**, 482–492 (1972).

GERMAN, B., WYMAN, J.: The titration curves of oxygenated and reduced haemoglobin. J. biol. Chem. **117**, 533–550 (1937).

GERSONDE, K., OVERKAMP, M., SICK, H., TRITTELVITZ, E., JUNGE, W.: β-chain allostery in the frozen quaternary T-structure of haemoglobin M Iwate. Eur. J. Biochem. In press (1973).

GIBSON, Q. H.: The contribution of the α- and β-chains to the kinetics of oxygen binding to and dissociation from hemoglobin. Proc. nat. Acad. Sci. (Wash.) **70**, 1–4 (1973).

GRAY, R. D.: The kinetics of the alkaline Bohr effect of human hemoglobin. J. biol. Chem. **245**, 2914–2921 (1970).

HABER, J. E., KOSHLAND, D. E.: The effect of 2,3-diphosphoglyceric acid on the changes in β–β interactions in hemoglobin during oxygenation. J. biol. Chem. **246**, 7790–7793 (1971).

HARMS, H., BARTELS, H.: CO_2-Dissoziationskurven des menschlichen Blutes bei Temperaturen von 5–37° C bei unterschiedlicher O_2-Sättigung. Pflügers Arch. **272**, 384–392 (1961).

HARTLEY, L. H.: Adjustments of the oxygen transport system during residence at high altitude. In: Hemoglobin and red cell structure and function. New York-London: Plenum Press 1972.

HASTINGS, A. B., VAN SLYKE, D. D., NEILL, J. M., HEIDELBERGER, M., HARINGTON, C. R.: Studies of gas and electrolyte equilibria in blood. IV. The acid properties of reduced and oxygenated hemoglobin. J. biol. Chem. **60**, 89–100 (1924).

HAUROWITZ, F.: Das Gleichgewicht zwischen Hämoglobin und Sauerstoff. Z. physiol. Chem. **254**, 266–274 (1938).

HAYASHI, A., SUZUKI, T., SHIMIZU, A., YAMAMURA, Y.: Properties of hemoglobin M. Unequivalent nature of the α- and β-subunits in the hemoglobin molecule. Biochim. Biophys. Acta **168**, 262–273 (1968).

HENRIQUES, O. M.: Die Bindungsweise des Kohlendioxyds im Blut. I. Über die Geschwindigkeit der Anhydrierung bzw. der Hydratisierung der Kohlensäurekomponenten im Blut. Biochem. Z. **200**, 1–9 (1928).

HENRIQUES, O. M.: Über Carbhämoglobin. Ergeb. Physiol. **28**, 625–689 (1929).

HENRY, Y., BANERJEE, R.: Electron paramagnetic studies of nitric oxide haemoglobin derivatives: Isolated subunits and nitric oxide hybrids. J. Mol. Biol. **73**, 469–482 (1973).

HENRY, Y., CASSOLY, R.: Chain non-equivalence in nitric oxide binding to hemoglobin. Biochem. Biophys. Res. Commun. **51**, 659–665 (1973).

HERMANN, H., HUDOFFSKY, B., NETTER, H., TRAVIA, L.: Über den spezifischen Einfluß der Kohlensäure auf die Sauerstoffbindungskurve des Hämoglobins. Pflügers Arch. **242**, 311–327 (1939).

HERZFELD, J., STANLEY, H. E.: A general model of cooperativity and its application to DPG inhibition of hemoglobin oxygenation. Biochem. Biophys. Res. Commun. **48**, 307–313 (1972).

HILL, A. V.: The possible effects of aggregation of the molecules of haemoglobin on its dissociation curve. J. Physiol. (Lond.) **40**, IV–VII (1910).

HILL, R. J., DAVIS, R. W.: The pK of specific groups of proteins. I. The α-amino group of the α-chain of human CO hemoglobin. J. biol. Chem. **242**, 2005–2012 (1967).

HILL, E. P., POWER, G. G., LONGO, L. D.: A mathematical model of carbon dioxide transfer in the placenta and its interaction with oxygen. Amer. J. Physiol. **224**, 283–299 (1973).

HOARD, J. L.: Stereochemistry of porphyrins. In: Hemes and hemoproteins. New York-London: Academic Press 1966.

HOPFIELD, J. J., SHULMAN, R. G., OGAWA, S.: An allosteric model of hemoglobin: I, Kinetics. J. Mol. Biol. **61**, 425–443 (1971).

HUESTIS, W. H., RAFTERY, M. A.: ^{19}F-NMR studies of oxygen binding to hemoglobin. Biochem. Biophys. Res. Commun. **49**, 1358–1365 (1972).

JOHANSEN, K., LENFANT, C.: A comparative approach to the adaptability of O_2-Hb affinity. In: Oxygen affinity of hemoglobin and red cell acid base status. Copenhagen: Munksgaard 1972.

KENDREW, J. C., DICKERSON, R. E., STRAUDBERG, B. E., HART, R. G., DAVIS, D. R., PHILLIPS, D. C., SHORE, V. C.: Structure of myoglobin. A three-dimensional Fourier synthesis at 2 Å resolution. Nature (Lond.) **185**, 422–427 (1960).

KEYS, A., HALL, F. G., GUZMAN BARRON, E. S.: The position of the oxygen dissociation curve of human blood at high altitude. Amer. J. Physiol. **115**, 292–307 (1936).

KILMARTIN, J. V., BREEN, J. J., ROBERTS, G. C. K., HO, C.: Direct measurement of the pK values of an alkaline Bohr group in human haemoglobin. Proc. nat. Acad. Sci. (Wash.) **70**, 1246–1249 (1973).

KILMARTIN, J. V., HEWITT, J. A.: The effect of removal of C-terminal residues on cooperative interactions in hemoglobin. Cold Spring Harbor Symp. Quant. Biol. **36**, 311–314 (1971).

KILMARTIN, J. V., ROSSI-BERNARDI, L.: Inhibition of CO_2 combination and reduction of the Bohr effect in haemoglobin chemically modified at its α-amino groups. Nature (Lond.) **222**, 1243–1246 (1969).

KILMARTIN, J. V., ROSSI-BERNARDI, L.: The binding of carbon dioxide by horse haemoglobin. Biochem. J. **124**, 31–45 (1971).

KILMARTIN, J. V., WOOTTON, J. F.: Inhibition of Bohr effect after removal of C-terminal histidines from haemoglobin β-chains. Nature (Lond.) **228**, 766–767 (1970).

KLINGER, R. G., ZAHN, D. P., BROX, D. H., FRUNDER, H. E.: Interaction of hemoglobin with ions. Binding of ATP to human hemoglobin under simulated in vivo conditions. Eur. J. Biochem. **18**, 171–177 (1971).

KLOCKE, R. A.: Mechanism and kinetics of the Haldane effect in human erythrocytes. J. appl. Physiol. In press (1973).

KOSHLAND, D. E., NEMETHY, G., FILMER, D.: Comparison of experimental binding data and theoretical models in proteins containing subunits. Biochemistry **5**, 365–385 (1966).

KREUZER, F., ROUGHTON, F. J. W., ROSSI-BERNARDI, L., KERNOHAN, J. C.: Specific effect of CO_2 and bicarbonate on the affinity of hemoglobin for oxygen. In: Oxygen affinity of hemoglobin and red cell acid base status. Copenhagen: Munksgaard 1972.

LENFANT, C., TORRANCE, J., ENGLISH, E., FINCH, C. A., REYNAFARJE, C., RAMOS, J., FAURA, J.: Effect of altitude on oxygen binding by hemoglobin and on organic phosphate level. J. clin. Invest. **47**, 2652–2656 (1968).

LENFANT, C., TORRANCE, J., REYNAFARJE, C.: Shift of the O_2-Hb dissociation curve at altitude; mechanism and effect. J. appl. Physiol. **30**, 625–631 (1971).

LINDSTROM, T. R., HO, C.: Functional nonequivalence of α and β hemes in human adult hemoglobin. Proc. nat. Acad. Sci. (Wash.) **69**, 1707–1710 (1972).

LINDSTROM, T. R., OLSON, J. S., MOCK, N. H., GIBSON, Q. H., HO, C.: Nuclear magnetic resonance studies of hemoglobin. VIII. Evidence for preferential ligand binding to β-chains within deoxyhemoglobins. Biochem. Biophys. Res. Commun. **45**, 22–26 (1971).

LO, H. H., SCHIMMEL, P. R.: Interaction of human hemoglobin with adenine nucleotides. J. biol Chem. **244**, 5084–5086 (1969).

LÜBBERS, D. W., LUFT, U. C., THEWS, G., WITZLEB, E.: Oxygen transport in blood and tissue. Stuttgart: Thieme 1971.

MACQUARRIE, R., GIBSON, Q. H.: Use of a fluorescent analogue of 2,3-diphosphoglycerate as a probe of human hemoglobin conformation during carbon monoxide binding. J. biol. Chem. **246**, 5832–5856 (1971).

MARÉES DE, H., BARBEY, K.: Änderung der peripheren Durchblutung durch Ausdauertraining. Z. Kardiol. **62**, 653–663 (1973).

MARGARIA, R., GREEN, A. A.: The first dissociation constant, pK, of carbonic acid in hemoglobin solutions and its relation to the existence of a combination of hemoglobin with carbon dioxide. J. biol. Chem. **102**, 611–634 (1933).

MCDONALD, M. J., NOBLE, R. W.: The effect of pH on the rates of ligand replacement reactions of human adult and foetal hemoglobins and their subunits. J. biol. Chem. **247**, 4282–4287 (1972).

METCALFE, J., DHINDSA, D. S.: The physiological effects of displacements of the oxygen dissociation curve. In: Oxygen affinity of hemoglobin and red cell acid base status. Copenhagen: Munksgaard 1972.

MILLER, M. E., RØRTH, M., PARVING, H. H., HOWARD, D., REDDINGTON, I., VALERI, C. R., STOHLMAN, F.: pH effect on erythropietin response to hypoxia. N. Engl. J. Med. **288**, 706–710 (1973).

MONOD, J., CHANGEUX, J.-P., JACOB, F.: Allosteric proteins and cellular control systems. J. Mol. Biol. **6**, 306–329 (1963).

MONOD, J., JACOB, F.: General conclusions: Teleonomic mechanisms in cellular metabolism, growth and differentiation. Cold Spring Harbor Symp. Quant. Biol. **26**, 389–401 (1961).

MONOD, J., WYMAN, J., CHANGEUX, J.-P.: On the nature of allosteric transitions: A plausible model. J. Mol. Biol. **12**, 88–118 (1965).

MOORE, L. G., BREWER, G. J., OELSCHLEGEL, F. J.: Red cell metabolic changes in acute and in chronic exposure to high altitude. In: Hemoglobin and red cell structure and function. New York-London: Plenum Press 1972.

MORROW, J. S., KEIM, P., VISSCHER, R. B., MARSHALL, R. C., GURD, F. R. N.: Interaction of $^{13}CO_2$ and bicarbonate with human hemoglobin preparations. Proc. nat. Acad. Sci. (Wash.) **70**, 1414–1418 (1973).

MUIRHAED, H., COX, J. M., MAZZARELLA, L., PERUTZ, M. F.: Structure and function of haemoglobin. III. A three dimensional Fourier synthesis of human deoxy-haemoglobin at 5.5 Å resolution. J. Mol. Biol. **28**, 117–156 (1967).

MUIRHAED, H., GREER, J.: Three dimensional Fourier synthesis of human deoxyhaemoglobin at 3.5 Å resolution. Nature (Lond.) **228**, 516–519 (1970).

MUIRHAED, H., PERUTZ, M. F.: Structure of haemoglobin. A three dimensional Fourier synthesis of reduced human haemoglobin at 5.5 Å resolution. Nature (Lond.) **199**, 633–638 (1963).

NIESEL, W.: Die Umlagerungshypothese. Ein Beitrag zur Klärung der Sauerstoff-Hämoglobin-Reaktion. Inauguraldissertation, Kiel (1961).

NOVY, M. J., EDWARDS, M. J., METCALFE, J.: Hemoglobin Yakima: II. High blood oxygen affinity associated with compensatory erythrocytosis and normal haemodynamics. J. clin. Invest. **46**, 1848–1854 (1967).

OLSON, J. S., GIBSON, Q. H.: The release of protons and anions during ligand binding to human deoxyhemoglobin. J. biol. Chem. **248**, 1623–1630 (1973a).

OLSON, J. S., GIBSON, Q. H.: The effects of pH and anions on the properties of the α- and β-chains within human deoxyhemoglobin. J. biol. Chem. **248**, 1616–1622 (1973b).

OGATA, R. T., MCCONNELL, H. M.: The binding of a spin-labeled triphosphate to hemoglobin. Cold Spring Harbor Symp. Quant. Biol. **36**, 325–335 (1971).

OGATA, R. T., MCCONNELL, H. M.: Mechanism of cooperative oxygen binding to hemoglobin. Proc. nat. Acad. Sci. (Wash.) **69**, 335–339 (1972a).

OGATA, R. T., MCCONNELL, H. M.: States of hemoglobin in solution. Biochemistry **11**, 4792–4799 (1972b).

OGAWA, S., MCCONNELL, H. M.: Spin-label study of hemoglobin conformations in solution. Proc. nat. Acad. Sci. (Wash.) **58**, 19–26 (1967).

OSKI, F. A., GOTTLIEB, A. J., DELIVORIA-PAPADOPOULOS, M., MILLER, W. W.: Red cell 2,3-diphosphoglycerate levels in subjects with chronic hypoxemia. N. Engl. J. Med. **280**, 1165–1166 (1969).

OSKI, F. A., MARSHALL, B. E., COHEN, P. J., SUGARMAN, H. J., MILLER, L. D.: Exercise with anemia. The role of the left-shifted or right-shifted oxygen-hemoglobin equilibrium curve. Ann. intern. Med. **74**, 44–46 (1971).

PACE, M., ROSSI-BERNARDI, L., ROUGHTON, F. J. W.: The effect of organic phosphates on the reactions of haemoglobin and oxyhaemoglobin (carboxy haemoglobin) with carbon dioxide. Biochem. J. **119**, 67–68P (1970).

PARER, J. T.: Oxygen transport in human subjects with hemoglobin variants having altered oxygen affinity. Resp. Physiol. **9**, 43–49 (1970).

PARER, J. T., JONES, W. D., METCALFE, J.: A quantitative comparison of oxygen transport in sheep and human subjects. Resp. Physiol. **2**, 196–206 (1967).

PAULING, L.: The oxygen equilibrium of hemoglobin and its structural interpretation. Proc. nat. Acad. Sci. (Wash.) **21**, 186–191 (1935).

PERUTZ, M. F.: Structure and function of haemoglobin. I. A tentative atomic model of horse oxyhaemoglobin. J. Mol. Biol. **13**, 646–668 (1965).

PERUTZ, M. F.: Stereochemistry of cooperative effects in haemoglobin. Nature (Lond.) **228**, 726–739 (1970).

PERUTZ, M. F.: Nature of haem-haem interaction. Nature (Lond.) **237**, 495–499 (1972).

PERUTZ, M. F., BOLTON, W., DIAMOND, R., MUIRHAED, H., WATSON, H. C.: Structure of haemoglobin. An X-ray examination of reduced horse haemoglobin. Nature (Lond.) **203**, 687–690 (1964).

PERUTZ, M. F., LEHMANN, H.: Molecular pathology of human haemoglobin. Nature (Lond.) **219**, 902–909 (1968).

PERUTZ, M. F., MAZZARELLA, L.: A preliminary X-ray analysis of haemoglobin H. Nature (Lond.) **199**, 639 (1963).

PERUTZ, M. F., MUIRHAED, H., COX, J. M., GOAMAN, L. C. G.: Three dimensional Fourier synthesis of horse oxyhaemoglobin at 2.8 Å resolution: the atomic model. Nature (Lond.) **219**, 131–139 (1968).

PERUTZ, M. F., MUIRHAED, H., MAZZARELLA, L., CROWTHER, R. A., GREER, J., KILMARTIN, J. V.: Identification of residues responsible for the alkaline Bohr effect in haemoglobin. Nature (Lond.) **222**, 1240–1243 (1969).

PERUTZ, M. F., ROSSMAN, M. G., CULLIS, A. F., MUIRHAED, H., WILL, G., NORTH, A. C. T.: Structure of haemoglobin. A three dimensional Fourier synthesis at 5.5 Å resolution obtained by X-ray analysis. Nature (Lond.) **185**, 416–422 (1960).

RAPOPORT, S.: Regulation of concentration of DPG and ATP in red blood cells. Försvarsmedicin **5**, 168–174 (1969).

RAPOPORT, S., GUEST, G. M.: Distribution of acid-soluble posphorus in the blood cells of various vertebrates. J. biol. Chem. **138**, 269–282 (1941).

RAPOPORT, S., MARETZKI, D., SCHEWE, C., JACOBASCH, G.: Control of glycolysis in the erythrocyte on the level of 1,3 DPG. In: Oxygen affinity of hemoglobin and red cell acid base status. Copenhagen: Munksgaard 1972.

RENTHAL, R., BENESCH, R. E., BENESCH, R., BRAY, B. A.: Affinity labeling of the polyphosphate binding site in hemoglobin with pyridoxal phosphate (PLP). Fed. Proc. **29**, 732 (1970).

RICHARDSON, T. Q., GUYTON, A. C.: Effects of polycythemia and anemia on cardiac output and other circulatory factors. Amer. J. Physiol. **197**, 1167–1170 (1959).

RIEGEL, K. P.: Respiratory gas transport characteristics of blood and hemoglobin. In: Physiology of the perinatal period. Vol. I. New York: Appleton-Century-Crofts 1970.

RIGGS, A.: Functional properties of hemoglobins. Physiol. Rev. **45**, 619–673 (1965).

RIGGS, A.: Mechanism of the enhancement of the Bohr effect in mammalian hemoglobins by diphosphoglycerate. Proc. nat. Acad. Sci. (Wash.) **68**, 2062–2065 (1971).

RØRTH, M.: Dependency on acid-base status of blood of oxyhemoglobin dissociation and 2,3-diphosphoglycerate level in human erythrocytes. I. In vitro studies on reduced and oxygenated blood. Scand. J. clin. Lab. Invest. **26**, 43–47 (1970).

RØRTH, M.: Hemoglobin interactions and red cell metabolism. Copenhagen: Thesis (1973).

RØRTH, M., NYGAARD, S. F., PARVING, H. H.: Red cell metabolism and oxygen affinity of healthy individuals during exposure to high altitude. In: Hemoglobin and red cell structure and function. New York-London: Plenum Press 1972.

ROSSI-BERNARDI, L., ROUGHTON, F. J. W.: The specific influence of carbon dioxide and carbamate compounds on the buffer power and Bohr effects in human haemoglobin solutions. J. Physiol. (Lond.) **189**, 1–29 (1967).

ROUGHTON, F. J. W.: Transport of oxygen and carbon dioxide. In: Handbook of Physiology. Respiration Vol. I. Washington D. C.: Am. Physiol. Soc. 1964.

ROUGHTON, F. J. W.: Some recent work on the interactions of oxygen, carbon dioxide and haemoglobin. Biochem. J. **117**, 801–812 (1970).

ROUGHTON, F. J. W., DELAND, E. C., KERNOHAN, J. C., SEVERINGHAUS, J. W.: Some recent studies of the oxyhaemoglobin dissociation curve of human blood under physiological conditions and the fitting of the Adair equation to the standard curve. In: Oxygen affinity of hemoglobin and red cell acid base status. Copenhagen: Munksgaard, 1972.

ROUGHTON, F. J. W., OTIS, A. B., LYSTER, R. L. J.: The determination of the individual equilibrium constants of the four intermediate reactions between oxygen and sheep haemoglobin. Proc. R. Soc. Lond. (Biol.) **144**, 29–54 (1955).

SALHANY, J. M., MATHERS, D. H., ELIOT, R. S.: The deoxygenation kinetics of hemoglobin partially saturated with carbon monoxide. J. biol. Chem. **247**, 6985–6990 (1972).

SALTIN, B., GAGGE, A. P., STOLWIJK, J. A. J.: Muscle temperature during submaximal exercise in man. J. appl. Physiol. **25**, 679–688 (1968).

SHULMAN, R. G., OGAWA, S., HOPFIELD, J. J.: An allosteric model of hemoglobin. Arch. Biochem. Biophys. **151**, 68–74 (1972).

SICK, H., GERSONDE, K., THOMPSON, J. C., MAURER, W., HAAR, W., RÜTERJANS, H.: The Bohr proton binding site in a monomeric haemoglobin. Eur. J. Biochem. **29**, 217–223 (1972).

SIEGFRIED, M.: Über die Bindung von Kohlensäure durch amphotere Amidokörper. Z. Phys. Chem. **44**, 85–96 (1905).

SIGGAARD-ANDERSEN, O.: Oxygen-linked hydrogen binding of human hemoglobin. Effects of carbon dioxide and 2,3-diphosphoglycerate. I. Studies on erythrolysate. Scand. J. clin. Lab. Invest. **27**, 351–360 (1971).

SIGGAARD-ANDERSEN, O., SALLING, N.: Oxygen-linked hydrogen binding of human hemoglobin. Effects of carbon dioxide and 2,3-diphosphoglycerate. II. Studies on whole blood. Scand. J. clin. Lab. Invest. **27**, 361–366 (1971).

SIGGAARD-ANDERSEN, O., SALLING, N., NÖRGAARD, B., RØRTH, M.: Oxygen-linked hydrogen binding of human hemoglobin. Effects of carbon dioxide and 2,3-diphosphoglycerate. III. Comparison of the Bohr effect and the Haldane effect. Scand. J. clin. Lab. Invest. **29**, 185–193 (1972).

SCHROEDER, W. A.: The hemoglobins. Ann. Rev. Biochem. **32**, 301–320 (1963).

SCHROEDER, W. A., JONES, R. T.: Some aspects of the chemistry and function of human and animal hemoglobins. Fortschr. Chem. Org. Naturst. **23**, 113–194 (1965).

SLYKE VAN, D. D., HASTINGS, A. B., MURRAY, C. D., SENDROY, J.: Studies of gas and electrolyte equilibria in blood. VIII. The distribution of hydrogen, chloride and bicarbonate ions in oxygenated and reduced blood. J. biol. Chem. **65**, 701–728 (1925).

STADIE, W. C., O'BRIEN, H.: The carbamate equilibrium. II. The equilibrium of oxyhemoglobin and reduced hemoglobin. J. biol. Chem. **117**, 439–470 (1937).

THORLING, E. B., ERSLEV, A. J.: The "tissue" tension of oxygen and erythropoiesis. Blood **31**, 332–343 (1968).

TOMITA, S., RIGGS, A.: Studies on the interaction of 2,3-diphosphoglycerate and carbon dioxide with hemoglobins from mouse, man and elephant. J. biol. Chem. **246**, 547–554 (1971).

TYUMA, I., SHIMIZU, K.: Different response to organic phosphates of human fetal and adult hemoglobins. Arch. Biochem. Biophys. **129**, 404–405 (1969).

TYUMA, I., SHIMIZU, K., IMAI, K.: Effect of 2,3-diphosphoglycerate on the cooperativity in oxygen binding of human adult hemoglobin. Biochem. Biophys. Res. Commun. **43**, 423–428 (1971).

VALERI, C. R., FORTIER, N. L.: Red cell 2,3-diphosphoglycerate and creatine levels in patients with red cell mass deficits or with cardio-pulmonary insufficiency. N. Engl. J. Med. **281**, 1452–1455 (1969).

VALERI, C. R., ZAROULIS, C. G., FORTIER, N. L.: Peripheral red cells as a functional biopsy to determine tissue oxygen tension. In: Oxygen affinity of hemoglobin and red cell acid base status. Copenhagen: Munksgaard 1972.

VARNAUSKAS, E., BJÖRNTORP, P., FAHLEN, M., PREROVSKY, I., STENBERG, J.: Effects of physical training on exercise blood flow and enzymatic activity in skeletal muscle. Cardiovasc. Res. **4**, 418–422 (1970).

VERDIER DE, C. H., GARBY, L.: Low binding of 2,3-diphosphoglycerate to haemoglobin F. A contribution to the knowledge of the binding site and an explanation for the high oxygen affinity of foetal blood. Scand. J. clin. Lab. Invest. **23**, 149–151 (1969).

VERSMOLD, H., BRAUSER, B.: Improved cellular oxygenation by 2,3-diphosphoglycerate: Quantitative measurement of tissue hypoxia by registration of absorption spectra of cytochrome a and hemoglobin in the intact organ. In: Erythrocytes, Thrombocytes, Leucocytes. Stuttgart: Thieme 1973.

VERSMOLD, H., WENNER, J., RIEGEL, K.: Changes of blood oxygen affinity and capacity and red cell 2,3 DPG evoked by exchange transfusions with ACD preserved blood in newborn infants: their interrelationship and influences on oxygen supply of tissues and erythropoiesis. Z. Kinderheilk. **113**, 1–18 (1972).

VIALE, R. O., MAGGIORA, G. M., INGRAHAM, L. M.: Molecular orbital evidence for Weiss's oxyhaemoglobin structure. Nature (Lond.) **203**, 183–184 (1964).

WANG, J. H.: Hemoglobin studies. II. A synthetic material with hemoglobin-like property. J. Am. Chem. Soc. **80**, 3168–3169 (1958).

WANG, J. H., NAKAHARA, A., FLEISCHER, E. B.: Hemoglobin studies. I. The combination of carbon monoxide with hemoglobin and related model compounds. J. Am. Chem. Soc. **80**, 1109–1113 (1958).

WEBER, G.: Ligand binding and internal equilibria in proteins. Biochemistry **11**, 864–878 (1972).

WEISS, J. J.: Nature of the iron-oxygen bond in oxyhaemoglobin. Nature (Lond.) **202**, 83–84 (1964).

WHITEHAED, E.: The regulation of enzyme activity and allosteric transition. Progr. Biophys. Mol. Biol. **21**, 321–397 (1970).

WITTENBERG, J. B., WITTENBERG, B. A., PEISACH, J., BLUMBERG, W. E.: On the state of the iron and the nature of the ligand in oxyhaemoglobin. Proc. nat. Acad. Sci. (Wash.) **67**, 1846–1853 (1970).

WOODSON, R. D., TORRANCE, J. D., SHAPPELL, S. D., LENFANT, C.: The effect of cardiac disease on hemoglobin oxygen binding. J. clin. Invest. **49**, 1349–1356 (1970).

WRANNE, B., WOODSON, R. D., DETTER, J. C.: Bohr effect: interaction between H^+, CO_2, and 2,3-DPG in fresh and stored blood. J. appl. Physiol. **32**, 749–754 (1972).

WYMAN, J.: Heme proteins. Adv. Protein Chem. **4**, 407–531 (1948).

WYMAN, J.: Allosteric effects in hemoglobin. Cold Spring Harbor Symp. Quant. Biol. **28**, 483–489 (1963).

WYMAN, J.: Allosteric linkage. J. Am. Chem. Soc. **89**, 2202–2218 (1967).

WYMAN, J., ALLEN, D. W.: The problem of the heme interactions in hemoglobin and the basis of the Bohr effect. J. Polym. Sci. **7**, 499–518 (1951).

ZUCKERKANDL, E., PAULING, L.: Molecular disease, evolution and genetic heterogeneity. In: Horizons in Biochemistry. New York-London: Academic Press 1962.

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The Interrelationship between Sodium and Calcium Fluxes across Cell Membranes*

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Contents

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I. Introduction

The classical experiments of RINGER (1883) were the first to provide evidence that calcium plays a role in physiological processes. In recent years a considerable body of data on the cellular actions of Ca has been accumulated, and numerous reviews have appeared. It is now recognized that Ca may play a fundamental role in cellular respiration (*e.g.* LEHNINGER et al., 1967; CARAFOLI and ROSSI, 1971). Calcium ions are required for muscular contraction (*e.g.* EBASHI and ENDO, 1968; COSTANTIN, 1974), and for the coupling of excitation to secretion in a large variety of secretory cells (*e.g.* DOUGLAS, 1969; RUBIN, 1970) and at nerve endings (*e.g.* KATZ, 1969). They are necessary for the maintenance of excitable membrane "stability" (*e.g.* SHANES, 1958a, b; BIANCHI, 1968), and in many types of excitable cells, Ca ions carry much of the inward current associated with depolarization (*e.g.* REUTER, 1973). Finally, Ca has a significant influence on membrane permeability: raising intracellular Ca uncouples intercellular connections (LOEWENSTEIN et al., 1967; OLIVEIRA-CASTRO and LOEWENSTEIN, 1971; LOEWENSTEIN, 1973) and increases potassium permeability in many types of cells (*e.g.* GARDOS, 1959; KREGENOW and HOFFMAN, 1971; ROMERO and WHITTAM, 1971; MEECH, 1972; KRNJEVIC and LISIEWICZ, 1972; JANSEN and NICHOLLS, 1973).

Despite this wealth of information on the physiological actions of Ca, relatively little is known about the mechanisms which govern the movements of Ca across cellular membranes, and which control the intracellular free Ca^{2+} concentration in resting cells. During the past few years data from a number of laboratories have provided evidence that in many types of cells the transport of Ca may involve, at least in part, an exchange of sodium ions for Ca ions. The primary objective of this article is to review these data and to discuss the possible mechanisms involved. In many instances the available information is rather fragmentary, and sometimes controversial. Most of these observations are, however, consistent with the idea that in many types of cells some of the energy for "active" Ca transport may be derived from the transmembrane Na electrochemical gradient, rather than from adenosine triphosphate (ATP) directly as is the case for the Na–K coupled pump (*e.g.* CALDWELL, 1969; DE WEER, 1973). Thus, the possible role of the Na gradient in Ca transport may be compared with the postulated role of the Na gradient in non-electrolyte transport (*e.g.* SCHULTZ and CURRAN, 1970; GIBB and EDDY, 1972; but see POTASHNER and JOHNSTONE, 1971; SCHAFER and HEINZ, 1971; and GECK et al., 1972).

A number of reviews, monographs and compendia on various aspects of calcium transport and metabolism have appeared in recent years, including those

by BRINK (1954), NEUMAN and NEUMAN (1958), SCHOFFENIELS (1963), WASSERMAN (1963), BORLE (1967), BIANCHI (1968), COMAR and BRONNER (1969), NICHOLS and WASSERMAN (1971), and BAKER (1972). Only the review by BAKER, which was limited to calcium metabolism in nerve, has included a discussion of coupled movements of Na and Ca.

II. Compartmentation of Sodium and Calcium

A. Extracellular Sodium and Calcium

As a first step in evaluating the role of an ion transport mechanism in terms of its contribution to cellular homeostasis, it is necessary to obtain information about the transmembrane electrochemical gradient(s) for the transported ion(s). This information is useful not only as a measure of whether or not an ion is distributed in accordance with the Gibbs-Donnan equilibrium (DONNAN, 1925); but also in determining the minimum energy requirements for maintenance of the steady-state electrochemical gradient in the event of a non-equilibrium distribution (cf. CALDWELL, 1969).

The situation for monovalent cations, at least in extracellular fluids, is relatively straightforward: there is general agreement that sodium and potassium are virtually completely ionized, as indicated by ion activity measurements (FRIEDMAN et al., 1963). The determination of ion activities for divalent cations, however, is complicated by the fact that these ions readily bind to proteins and complex with small polyvalent anions. Furthermore, since the constants for complex formation have generally been determined under idealized *in vitro* conditions, errors may be introduced when these constants are used to evaluate ion interactions in physiological fluids (see below). This uncertainty may be minimal in the case of human blood plasma, where both chemical (NEUMAN and NEUMAN, 1958; WALSER, 1961) and bioassay (MCLEAN and HASTINGS, 1935) data indicate that slightly less than half the plasma calcium is ionized. According to WALSER (1961), of the total plasma Ca (2.4 mmoles/liter), about 47 per cent is ionized and about 7 per cent is complexed to polyvalent anions (including primarily phosphate and citrate); the remaining 46 per cent is not ultrafilterable, and is therefore presumably protein-bound. By comparison, in human cerebrospinal fluid, which has a much lower protein concentration than plasma, nearly all of the Ca is ultrafilterable, and most of it is likely ionized (PAUPE, 1957; MCLEAN and HASTINGS, 1935).

Similar detailed analyses of Ca binding and complex formation in plasma are not available for other higher vertebrates. However, data on the total calcium, protein and polyvalent anion concentrations in the blood plasma of other mammals (and even birds, reptiles and amphibians; DITTMER, 1961) suggest that the pattern of Ca binding and complexation in human blood plasma may be fairly representative of this group.

By way of comparison, marine invertebrates probably exhibit a somewhat different pattern. To begin with, the hemolymph of most marine arthropods and molluscs for which data are available (DITTMER, 1961) have total Ca concentrations of the order of 8–12 mM, considerably higher than the typical plasma Ca concentrations of higher vertebrates (2–5 mM; DITTMER, 1961).

Little is known about the Ca-binding properties of the hemolymph protein of marine invertebrates; data from our own laboratory (BLAUSTEIN, unpublished) indicate that about 90–93 per cent of the Ca in barnacle (*Balanus nubilus*) and squid (*Loligo pealei*) hemolymph is dializable. There is, however, reason to believe that a significant fraction of the Ca is complexed to polyvalent anions. Although data about inorganic phosphate concentrations is scanty (cf. DITTMER, 1961, page 296), the relatively high concentration of sulfate in the hemolymph of a number of marine molluscs (about 28 mmoles/liter in squid hemolymph; HAYES and PELLUET, 1947) may indicate that a significant fraction of the hemolymph Ca is in the form of undissociated $CaSO_4$. Most of the sulfate must, however, be in the form of undissociated $MgSO_4$, in view of the high concentration of Mg in the hemolymph (50 mmoles/liter; BLAUSTEIN, unpublished data), and the fact that the dissociation constants for $CaSO_4$ and $MgSO_4$ are about the same – about 5×10^{-3} Molar^{-1} (SILLEN, 1964). Taking values of 50 mmoles/liter for total Mg and 7 mmoles/liter for dializable Ca in squid (*Loligo pealii*) hemolymph (BLAUSTEIN, unpublished data, and see HAYES and PELLUET, 1947), the concentration of free Ca^{2+} in squid blood can be calculated by assuming that 12 per cent of the undissociated divalent sulfate ($M^{2+}SO_4$) is $CaSO_4$ (since 12 per cent of the divalent cation is Ca):

$$K_{M^{2+}SO_4} = \frac{([Mg^{2+}] + [Ca^{2+}])\,([SO_4^{2-}])}{[MgSO_4] + [CaSO_4]} = 5 \times 10^{-3}\ M^{-1}. \qquad (1)$$

According to Equation (1), the free, ionized Ca^{2+} in squid hemolymph should be about 4 mmoles/liter. This value may be an over-estimate if some of the Mg is protein-bound, or if other undissociated salts of Ca and/or Mg are present (such as the phosphates or carbonates).

One more factor to be considered in describing the state of Ca in extracellular fluids is the activity coefficient for this ion. Since we are dealing here not with simple binary electrolytes, but rather, with complex solutions which include a variety of ions, it is important to recall the dictum of LEWIS and RANDALL (1921): "In dilute solutions (*i.e.* ionic strengths of 'a few hundredths to a few tenths') the activity coefficient of any ion depends solely upon the total ionic strength of the solution". Accordingly, we have used the Debye-Hückel equation, as modified by GÜNTELBERG and GUGGENHEIM (ROBINSON and STOKES, 1968, page 231; and see SHATKAY, 1968) to calculate the single ion activity coefficient for Ca^{2+} (f_{Ca}) in mixed electrolytes resembling human blood plasma and squid hemolymph (see Appendix). These values, 0.30 and 0.23, respectively (Table 1), are within 15 per cent of the Ca^{2+} ion activity coefficients in pure $CaCl_2$ solutions calculated from hydration theory (BATES et al., 1970).

The main point about the data in Table 1 is that they indicate that the activities of Ca^{2+} in vertebrate blood plasma and in marine invertebrate hemolymph are about 1/300th of the activities of Na in the same solutions.

Table 1.

	Ionic Strength (I)	Free Ca^{+2} Concentration (c_{Ca}) moles/liter	Na^+ Concentration (c_{Na}) moles/liter	Debye-Hükel constants		
				A	b	
					NaCl	$CaCl_2$
Human blood plasma	0.15	1.2×10^{-3}	0.145	0.52	0.10	0.20
Squid hemolymph	0.58	4×10^{-3}	0.437	0.50	0.10	0.20

	Sodium activity coefficients ($f_{Na}=f_{Cl}$)	Calcium activity coefficients (f_{Ca})	Sodium activities[a] (a_{Na}) moles/liter	Calcium activities[a] (a_{Ca}) moles/liter
Human blood plasma	0.74	0.30	0.11	3.6×10^{-4}
Squid hemolymph	0.70	0.23	0.30	9.3×10^{-4}

[a] The activity, a_i is equal to $f_i \times c_i$, where c_i is the (free) molar concentration of the i^{th} ion.

B. Intracellular Sodium and Calcium

Considerable controversy surrounds the question of whether or not the alkali metal ions in the cytoplasm are bound to cytoplasmic proteins. For example, although some nuclear magnetic resonance data have been interpreted as indicating considerable binding of intracellular Na (COPE, 1970), alternative interpretations have also been put forth (SHPORER and CIVAN, 1972). However, HINKE (1961), using ion-specific microelectrodes in the squid giant axon, provided evidence that about 76 per cent of the Na and virtually all of the K in the axoplasm is free. MCLAUGHLIN and HINKE (1966) applied similar techniques to giant barnacle muscle fibers and concluded that a significant fraction of the intracellular Na (about 84 per cent), but none of the sarcoplasmic K, was bound. Again, there is not unanimity of opinion on this point, but much of the controversy here concerns the magnitude of the total intracellular Na concentration in barnacle muscle (cf. GAYTON et al., 1969).

The distribution of Ca within cells presents an entirely different problem: although the total Ca in many vertebrate cells is of the order of 0.5–2 mmoles/liter fiber water, there is general agreement that, in most cells which have been studied, only a small fraction of the intracellular Ca is present as free ions. Much of the cell Ca is normally sequestered in organelles, such as mitochondria (see below). In relaxed muscle, a considerable fraction of the cell Ca is stored in the sarcoplasmic reticulum (e.g. WEBER, 1966; EBASHI et al., 1969).

In view of this sequestration, and of the possibility of binding and complex formation, the main unresolved question is: How much of the intracellular Ca is present as free Ca^{2+}? An answer is lacking for most vertebrate tissues primarily because of technical limitations. Very crude estimates of intracellular Ca distri-

bution have been made by studying the subfractionation of Ca and protein in homogenates of liver (CARAFOLI, 1967), brain (TOWER, 1968) and intestinal mucosa (CASSIDY et al., 1969). The highest concentrations of Ca (per gram protein) are found in the mitochondria, and only about 1–10 per cent of the total tissue Ca remains in the clear supernatant solution following high-speed centrifugation.

Mitochondria isolated from a variety of mammalian tissue sources exhibit a high-affinity Ca uptake mechanism (e.g. LEHNINGER, 1970; CARAFOLI and ROSSI, 1971). When rat liver mitochondria are incubated with an equimolar mixture of Ca and adenosine diphosphate (ADP), no phosphorylation of the ADP occurs until the Ca^{2+} concentration of the medium is decreased to about 2×10^{-6} M by the respiration-linked mitochondrial accumulation of Ca (ROSSI and LEHNINGER, 1964; DRAHOTA et al., 1965; and see LEHNINGER, 1970). These data may indicate that the ionized Ca^{2+} concentration in the cytosol of intact cells may have to be about 2 μM or less in order to permit oxidative phosphorylation.

A clearer picture emerges from studies of skeletal muscle. Since the contractile processes require Ca^{2+} ions in low concentration (e.g. EBASHI and ENDO, 1968), the Ca^{2+} concentration at the contraction threshold provides a lower limit for the pCa[1] in relaxed muscle fibers – about 6.3–7.0 (FILO et al., 1965; HELLAM and PODOLSKY, 1969; JULIAN, 1971).

The giant nerve and muscle fibers of certain marine molluscs and arthropods have been particularly useful sources of information about the distribution of intracellular Ca. PORTZEHL et al. (1964) injected solutions containing Ca buffered with EGTA [ethylene glycol bis (β-aminoethyl ether)-N,N′-tetraacetate] into single muscle fibers of the crab, *Maia squinado*. Their data enabled them to calculate that the pCa at the contraction threshold was 5.8–6.5. Likewise, HAGIWARA and NAKAJIMA (1966) examined the effect of injected Ca-EGTA buffers on the excitability of giant barnacle (*Balanus nubilus*) muscle fibers; from these data they concluded that the pCa in the sarcoplasm of resting fibers was between 6.1 and 6.7. The apparent binding constant of EGTA for Ca used to derive these values was calculated from the data of SCHWARTZENBACH et al. (1957). However, as pointed out by EBASHI (1962) and OGAWA (1968), these calculated binding constants may be more than an order of magnitude higher than EGTA binding constants obtained experimentally under physiological conditions; consequently, the free Ca^{2+} concentrations may be somewhat underestimated in these muscle studies (but see JULIAN, 1971).

The state of Ca in the axoplasm of squid giant axons has been the subject of several investigations. Although these have recently been reviewed by BAKER (1972), a few of the salient features will be mentioned here for the convenience of the reader, because they are pertinent to some of the discussion which follows. HODGKIN and KEYNES (1957) first studied the diffusion and mobility of Ca in squid axons, following the injection of small patches of ^{45}Ca into the axoplasm. They obtained a mean value of 1.2×10^{-6} cm²/sec for the diffusion coefficient, but stated that the lowest values (0.6×10^{-6} cm²/sec), obtained in their longest experiments, were probably the most reliable. The latter value is an order of magnitude less than the self-diffusion coefficient for Ca in sea water. The Ca

[1] pCa is the negative logarithm (base 10) of the molar intracellular free Ca^{2+} concentration.

mobility was too small to be measured with precision, but the upper limit was estimated to be 0.9×10^{-5} cm/sec per volt/cm, a value only 1/45th of that in free solution. This result suggests that, at most, only about 2 per cent (or less than 10 μM) of the total axoplasmic Ca (0.4 mM – KEYNES and LEWIS, 1956; BLAUSTEIN and HODGKIN, 1969) is ionized (pCa > 5). LUXORO and YAÑEZ (1968) and BLAUSTEIN and HODGKIN (1969) measured the efflux of Ca from axoplasm which was injected with ^{45}Ca and then extruded into dialysis bags; the results of these experiments also indicated that slightly less than 1/50th of the axoplasmic Ca was free.

KATZ and MILEDI (1967; 1969; 1970) measured post-synaptic depolarization as an indication of depolarization-induced presynaptic Ca conductance increase, Ca influx and transmitter release at the squid giant synapse (see Section III B 2b). Moderate presynaptic depolarization normally evoked post-synaptic depolarization. But with very large presynaptic depolarizations, to +130 mV or more, large post-synaptic depolarizations were observed only when the presynaptic membrane was repolarized. This suggests that the presynaptic Ca conductance was increased by depolarization, but that perhaps Ca^{2+} could not carry inward current because the membrane was depolarized to, or beyond, the Ca equilibrium potential (E_{Ca}; see Equation 2). Assuming that the "suppression potential" (+130 mV) was approximately equal to E_{Ca}, KATZ and MILEDI (1967) calculated that pCa in the squid axoplasm was about 6.4.

More recently, BAKER et al. (1971) employed the photoprotein, aequorin, to measure the resting free Ca^{2+} concentration in squid axoplasm. Aequorin, which emits visible light in the presence of Ca^{2+}, was first injected into the axon, and the "resting glow" was measured. Subsequently Ca-EGTA buffers were injected in an effort to alter or maintain the resting glow. A buffer containing about 45 mM Ca and 100 mM EGTA was found to maintain the resting glow. From these data, BAKER et al. concluded that if the ionized intracellular magnesium concentration is about 10 mM, the minimum pCa in the axoplasm is about 6.5. However, this value, again, is based upon the apparent binding constant (K_{app}) of EGTA for Ca obtained from the data of SCHWARTZENBACH et al. (1957); if the K_{app} is taken as $4 \times 10^5\ M^{-1}$ (EBASHI, 1961; OGAWA, 1968), a value of about 5.5–5.7 is obtained for the axoplasmic pCa.

Irrespective of whether the correct value for free Ca^{2+} in squid axoplasm is in the micromolar or tenth-micromolar range (pCa = 5–6 or 6–7, respectively), it is clear that at least 98 per cent of the Ca in axoplasm is not free. Experiments on the effects of metabolic poisons on the Ca efflux from ^{45}Ca-loaded axons (ROJAS and HIDALGO, 1968; BLAUSTEIN and HODGKIN, 1969), and on the light emission of axons injected with aequorin (BAKER et al., 1971) indicate that most of this unionized Ca is sequestered by an ATP-dependent process. Fig. 1 shows that the Ca efflux from a ^{45}Ca-injected axon increased dramatically (10-fold in this case), following a 60–90 minute delay, when the fiber (solid symbols) was exposed to cyanide. The delay is presumably a reflection of the time required for the axoplasmic ATP concentration to fall sufficiently (CALDWELL, 1960). The increased ^{45}Ca efflux from the poisoned fiber appears to be the direct consequence of a markedly reduced pCa: The rate of loss of ^{45}Ca from the extruded axoplasm of the unpoisoned axon (open symbols) was initially very low, and only increased with a

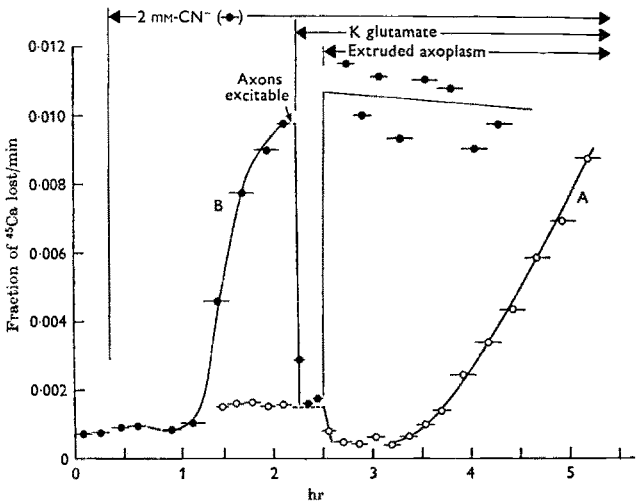


Fig. 1. Ca efflux before and after extrusion in A (○) unpoisoned squid axon and B (●) cyanide-poisoned axon. Abscissa: time. Ordinate: rate constant of Ca efflux. Axon diameters: A, 1030 μ, and B, 840 μ. (From BLAUSTEIN and HODGKIN, 1969)

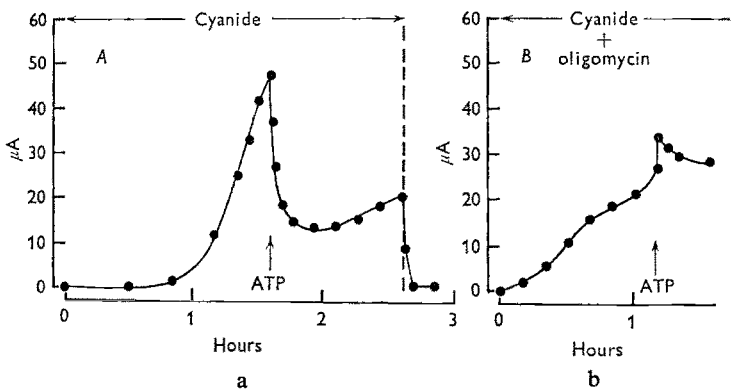


Fig. 2a and b. Injection of ATP into cyanide-poisoned squid axons. In a, the axon was exposed to artificial sea water (ASW) containing 2 mM-CN at zero time and was returned to cyanide-free ASW at the time marked by a vertical dashed line. In b, the axon was pre-treated for 30 min with oligomycin both by injection to give a final concentration of 50 μg/ml and also inclusion of 5 μg/ml in the ASW. At zero time 2 mM-CN was added to the oligomycin-ASW bathing the axon. In both a and b, ATP was injected at the vertical arrows, to give a final concentration of 30 μM. Both axons contained 0.18 μl aequorin. Diameter of axon A, 650 μ, and B, 850 μ. Temp. 21° C. (From BAKER et al., 1971)

delay which is likely due to the eventual loss of substrate to fuel the sequestration process (cf. BLAUSTEIN and HODGKIN, 1969).

More direct evidence that cyanide reduces pCa is given in Fig. 2a, which shows that in an aequorin-injected axon the light emission greatly increased about an hour after the introduction of cyanide. Subsequent injection of ATP significantly reduced the light emission (Fig. 2a), presumably by fueling the re-sequestration of Ca. However, injection of ATP had little effect on the luminescence which

occurred in the presence of both cyanide and oligomycin (Fig. 2b). This may implicate mitochondria as the primary site of Ca sequestration in squid axoplasm since accumulation of Ca by mitochondria may proceed either via cyanide-sensitive oxidative metabolism, or via an ATP-dependent, oligomycin-sensitive route (e.g. CARAFOLI and ROSSI, 1971).

In sum, the pCa of squid axoplasm is probably in the range of 5.5–6.5, and most of the intracellular Ca is sequestered in ATP-dependent stores-presumably mitochondria.

Finally, the activity coefficient for intracellular Ca should also be mentioned. Its value is probably quite similar to the f_{Ca} in the extracellular fluid. However, the fact that unionized taurine may account for 5–10 per cent of the osmotically active solute in the axoplasm (KOECHLIN, 1955) may mean that, with a lower ionic strength, f_{Ca} will be slightly higher here than in the hemolymph.

C. The Electrochemical Gradient for Calcium

In order for Ca to be distributed at equilibrium across the plasma membrane, the Ca diffusion potential (E_{Ca}), as given by the Nernst relation[2]:

$$E_{Ca} = \frac{RT}{2F} \ln \frac{a_{Ca_o}}{a_{Ca_i}} \tag{2}$$

should be equal to the resting membrane potential. With a resting potential of the order of -60 mV (cytoplasm negative), as observed in many nerve and muscle cells, the Nernst relation predicts that the intracellular Ca activity (^aCa_i) should be about 100-fold greater than the extracellular activity (^aCa_o), rather than a small fraction of it—as is actually the case. Similar reasoning may be applied to most cell types in higher animals, since a negative membrane potential is the general rule. This leads to the conclusion that the steady-state distribution of Ca is far from equilibrium in most animal cells, and, if the cell membranes are not totally impermeable to Ca, these cells must be capable of extruding Ca against a large electrochemical gradient. Consequently, active transport of Ca must play a particularly critical role in the recovery process of those tissues (e.g. certain nerve, muscle and glandular cells) in which activity is associated with the net entry of Ca ions (see DOUGLAS, 1968; RUBIN, 1970; REUTER, 1973). The subsequent sections of this article are concerned with the problem of "active" Ca transport, with particular emphasis on the possible role of Na–Ca exchange in this process.

III. Calcium Fluxes in Nerve

A. Invertebrate Nervous Tissue

1. The Squid Axon

Squid axons, during electrical activity, normally gain a small amount of Ca (HODGKIN and KEYNES, 1957; BAKER et al., 1971). Although the functional signi-

[2] Where R is the gas constant, T is absolute temperature and F is Faraday's number.

ficance of this Ca influx is not understood, it is readily apparent that during recovery there must be a net extrusion of Ca against a large electrochemical gradient.

a) Calcium Efflux

In considering the efflux of a cation, we would like to obtain answers to several pertinent questions, including: 1. Is the efflux of the cation accompanied by an anion? or 2. Does the cation exchange for another cation, moving in the opposite direction, with no net movement of charge incurred? or 3. Is the cation efflux electrogenic, in as much as the coupled anion or cation movements (if they occur) are insufficient (or over-sufficient) to balance the charge transfer of the cation efflux of interest? And, since we are concerned with an "uphill" movement of Ca, 4. What is the immediate source of energy for this "active" transport process?

The information obtained from experiments on squid axons have provided some of the most definitive (albeit by no means complete) answers to these questions. It is therefore most convenient to begin with a discussion of these data, and then to compare them with the results obtained from a variety of other preparations.

Several characteristic features of the Ca efflux from squid axons, which bear on the aforementioned questions, are illustrated in Fig. 3. There is, in the first place, a dependency on certain cations in the external medium: in the unpoisoned axon, when external Ca is replaced by Mg, the Ca efflux is reduced by about 35 per cent. A further 25 per cent reduction in efflux (from the Ca-free baseline) occurs when Li, choline or dextrose is substituted for external Na (BLAUSTEIN and HODGKIN, 1969; and see Fig. 3). The most straight forward explanation for these observations is that a significant fraction of the Ca efflux involves an exchange[3] for Ca_o and Na_o. In one experiment, the Ca efflux was reduced to about 10 per cent of its value in control sea water when all of the external Na, Ca and Mg were replaced by choline (BLAUSTEIN and HODGKIN, 1969). On the face of it, this might be construed as being indicative of a Ca–Mg exchange; the issue is complicated, however, by the fact that the squid axon may depolarize in a divalent cation-free solution (cf. SHANES, 1958a; FRANKENHAEUSER and HODGKIN, 1957), and depolarization may itself reduce the Ca efflux (see below). Furthermore, in the presence of external Ca and Na, removal of external Mg has little, if any, effect on Ca efflux (BAKER and CRAWFORD, 1972). Consequently, the Na_o- and Ca_o-independent efflux remains as something of an enigma, and will be reconsidered below.

In experiments in which Na_o was replaced by Li, in the presence of Ca_o, the loss of ^{45}Ca also decreased, but recovery was incomplete when Na_o was reintroduced (BLAUSTEIN and HODGKIN, 1969). One explanation for the apparent decline of the Ca efflux and the incomplete reversibility is that the Ca efflux mechanism may become saturated or inhibited by the large influx of Ca which occurs from Na-free, Li sea water (Section III, A, 1b).

When the axoplasmic pCa is reduced by poisoning the axon with cyanide (Section II, B), the attendant increase in Ca efflux is largely dependent upon

[3] Subscripts "o" and "i" refer to external and intracellular environments, respectively. Square brackets around a chemical symbol (e.g. [Na]) refer to the concentration of the ion.

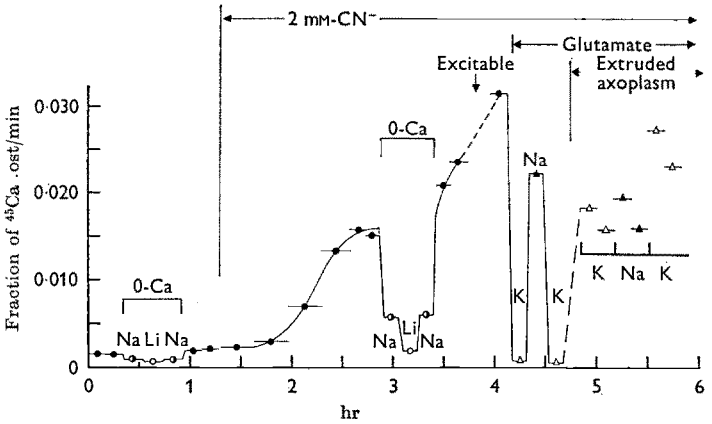


Fig. 3. Effect of removing first calcium and then sodium on Ca efflux in an unpoisoned squid axon and then during cyanide-poisoning. The second part of the figure shows the effect of K or Na glutamate on Ca efflux from the intact axon and from extruded axoplasm. Abscissa: time. Ordinate: rate constant of Ca efflux. ● Standard (artificial) Na sea water. ◑ Ca replaced by Mg. ○ Ca replaced by Mg and Na replaced by Li. △ Sea water replaced by Ca-free and Mg-free K glutamate. ▲ Same but K replace by Na. (From BLAUSTEIN and HODGKIN, 1969)

external Ca and Na (Fig. 3). On the average, in poisoned axons, about 70 per cent of the total Ca efflux was dependent upon external Ca, and 80 per cent of the residual efflux in Ca-free sea water required the presence of Na_o. This observation may indicate that ATP is not required for Na–Ca exchange, since the ATP concentration falls to about 100 μM in the presence of cyanide (CALDWELL, 1960; MULLINS and BRINLEY, 1967)—a level sufficiently low to markedly inhibit the Na–K exchange pump (HODGKIN and KEYNES, 1955; MULLINS and BRINLEY, 1967). Furthermore, DIPOLO (1973a), using internal dialysis techniques (BRINLEY and MULLINS, 1967) to reduce the axoplasmic ATP concentration below 5 μM, did not observe significant inhibition of $Ca_o + Na_o$-dependent Ca efflux—nor was the magnitude of the $Ca_o + Na_o$-dependent efflux affected by the subsequent addition of ATP to the internal solution. However, the question of possible ATP involvement is not yet settled, as BAKER and GLITSCH (1973) have obtained evidence that at high intracellular pCa, reduction of ATP inhibits Ca efflux.

Returning to the question of external cation activation of the Ca efflux, Fig. 3 shows that extruded axoplasm loses ^{45}Ca irrespective of the species of cation (Na or K) present in the medium, whereas in the intact axon ^{45}Ca efflux was specifically Na_o-dependent. These data indicate that the mechanism which mediates the Na-dependent Ca efflux is located at the plasma membrane.

The quantitative relationship between the Ca efflux and the external Na concentration is shown in Fig. 4. The data describe a sigmoid curve which best fits a cube-law relationship (see Fig. 4 caption) and may indicate that three Na^+ ions enter the axon in exchange for one Ca^{2+} ion. Although these data were obtained from internal dialysis experiments on cyanide-poisoned axons with rather low pCa's (3.5–3.7), a virtually identical relationship was observed in intact, unpoisoned barnacle muscle fibers (RUSSELL and BLAUSTEIN, 1974; but see BAKER

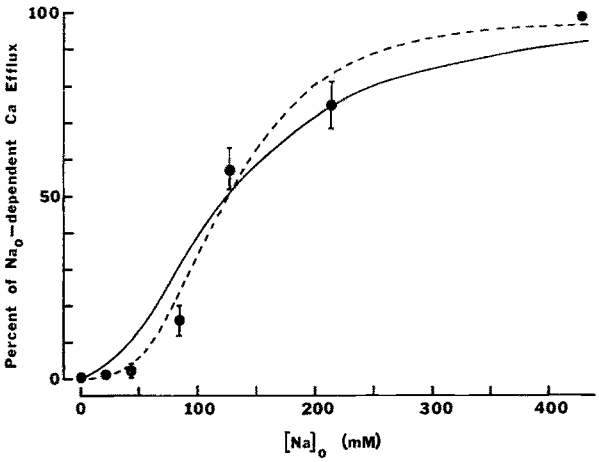


Fig. 4. Per cent of maximal Na_o-dependent ^{45}Ca efflux from internally-dialized squid (*Loligo pealei*) axons, graphed as a function of the external Na concentration. The data are mean values from five axons; the standard errors are indicated by the vertical bars. The axons were internally-dialized with an ATP-free solution containing 2 mM cyanide and a pCa of 3.5–3.7; the temperature for these experiments was 15 ± 1°C. The Na_o-dependent Ca efflux into Ca-free 425 mM Na-containing sea water, taken as 100% in the graph, averaged 2.78 ± 0.18 pmoles · cm^{-2} · sec^{-1}; the mean efflux into Ca-free, Na-free sea water was 0.05 ± 0.01 pmoles · cm^{-2} · sec^{-1}. Lithium was substituted isosmotically for Na in all of these experiments. The smooth curves were drawn to fit the equation:

$$v = \frac{V}{1 + \left(\frac{\bar{K}_{Na}}{[Na]_o}\right)^n}$$

where v is the Na_o-dependent ^{45}Ca efflux at any external Na concentration ($[Na]_o$), relative to that in Ca-free, Na-rich medium (V); $\bar{K}_{Na}$ is the apparent half-saturation constant for N_o, with a value of 125 mM. The exponent, n, had a value of 2 (solid line) or 3 (dashed line) (BLAUSTEIN et al., 1974).

and GLITSCH, 1973, who have evidence that the affinity for Na, and perhaps Ca, may be under metabolic control).

If the stoichiometry of the Na–Ca exchange is indeed 3 Na^+: 1 Ca^{2+}, one possibility is that the transport mechanism is electrogenic. In this case, for a complete transport cycle, the axon will gain one positive charge due to the entry of the third Na^+. If this inward-moving Na^+ acts as a counter-ion to a negative charge on the transport mechanism, the exit of Ca^{2+} might be accompanied by the free negative charge. Recent evidence indicating that the Na_o-dependent Ca efflux is sensitive to the electric field across the membrane, and is inhibited by depolarization (Fig. 5 and BRINLEY and MULLINS, personal communication) and enhanced by hyperpolarization (BRINLEY and MULLINS, personal communication) is consistent with this idea. In quantitative terms, the Ca efflux is reduced e-fold (where e is the base of the natural logarithms), or a little less, by a 25 mV depolarization (Fig. 6 and BRINLEY and MULLINS, personal communication). This reduction fits with the hypothesis that Ca exits in the company of one free negative charge (or that the return limb of the cycle brings in a free positive charge).

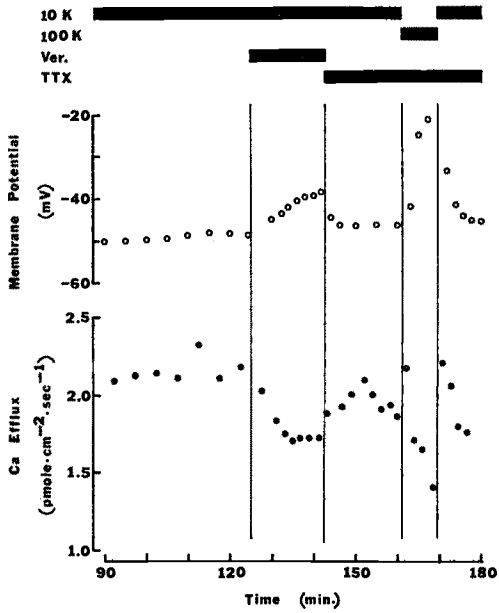


Fig. 5. Effect of membrane potential on ^{45}Ca efflux (solid circles, lower portion of figure) from an internally-dialized squid axon. The time in icated on the abscissa refers to the elapsed time from the start of the internal dialysis. The membrane potential, recorded from the end of the dialysis tube, is shown in the middle part of the figure. The bars at the top indicate the presence in the external medium of either 10 mM K (10 K) or 100 mM K (100 K), and of 1.5×10^{-4} M veratridine (Ver) or 10^{-6} M tetrodotoxin (TTX). The veratridine and K-rich solutions were used to depolarize the axon; TTX is known to reverse veratridine-induced depolarization in squid axons (OHTA et al., 1973). Axon 6013b; Diam. 630 µ; Temp. 15° C. The internal dialysis fluid was ATP-free and contained 2 mM cyanide, 50 mM Na, 350 mM K and had a pCa of 3.7 (BLAUSTEIN et al., 1974).

Fig. 7 provides further evidence that there is a site on the transport mechanism which accepts alkali metal ions, and is distinguishable from the site occupied by Ca. This experiment shows that the Ca_o-dependent Ca efflux is activated by Li, but not by choline; external Na can also activate the Ca_o-dependent Ca efflux (BLAUSTEIN, RUSSELL and DE WEER unpublished data). These data may indicate that in the Ca–Ca exchange mode (i.e. during Ca_o-dependent Ca efflux), entering Ca ions must be accompanied by either Na or Li (the two effective ions tested).

Since Ca efflux is at least in part dependent upon external Ca or Na, suggesting that Ca_i can exchange for either Ca_o or Na_o, it is possible that these ion exchanges may utilize a single transport mechanism containing some sites which can accommodate either ions species—but not both simultaneously. One might expect Na and Ca to compete for these sites, and indeed, Fig. 8 shows that increasing $[Na]_i$ inhibits Ca efflux—perhaps indicative of a displacement of Ca from the transport sites by Na. The curve is considerably flatter than anticipated if two Na^+ ions displace one Ca^{2+} (in which case the Ca efflux ought to be proportional to $1/[Na]^2$). However, these data were obtained at a relatively low (non-physiologic)

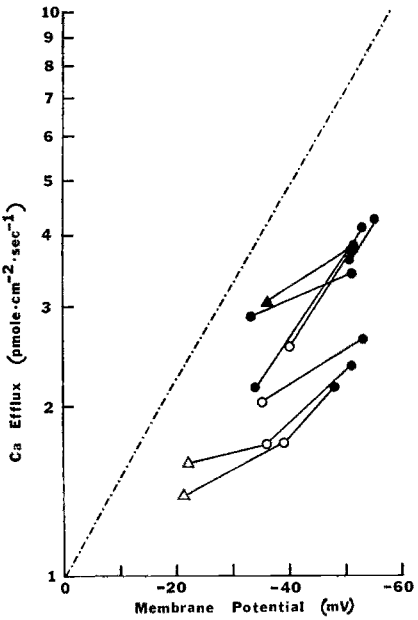


Fig. 6. Effect of membrane potential on ^{45}Ca efflux from internally-dialized squid axons. Data from 8 axons are summarized in the figure; data from individual axons are connected by solid lines. ^{45}Ca efflux into Ca-free sea water containing 10 mM K and 425 mM Na is indicated by solid circles. In some instances the axons depolarized spontaneously, as denoted by two solid circles connected by a line. In other instances 50 mM K (▲) or 100 mM K (△) or 5×10^{-4} M veratridine (○) was used to depolarize the axons. All axons were dialized with ATP-free solution containing 2 mM cyanide; the pCa was 3.5–3.7, and the temperature was $15 \pm 1°$ C. The broken line (-·-·) has a slope equivalent to an e-fold reduction in Ca efflux for a 25 mV depolarization BLAUSTEIN et al., 1974).

pCa (3.5–3.7), where the Ca concentration may tend to saturate the transport sites (DIPOLO, 1973a); unfortunately, there is no comparable data available for lower Ca concentrations.

b) Calcium Influx

There is also a competition between Na and Ca at the external surface of the membrane—most readily demonstrated in Ca influx experiments (BAKER et al., 1969): when external Na is replaced by Li or dextrose, Ca influx increases (Fig. 9a). There are, however, important quantitative differences between the effects of these two Na-substitutes. As Na is replaced by Li, Ca influx increases monotonically. With dextrose as a substitute, however, the Ca influx increases to a peak as $[Na]_o$ is reduced to about 100 mM, and further reduction of Na_o causes a fall in Ca influx. These observations complement the aforementioned Ca efflux studies by providing further evidence that the Ca transport mechanism contains two types of sites: one which can be occupied by either Ca or Na (presumably two Na^+), and a second type ("activator"), which accepts a single alkali metal ion, and does not

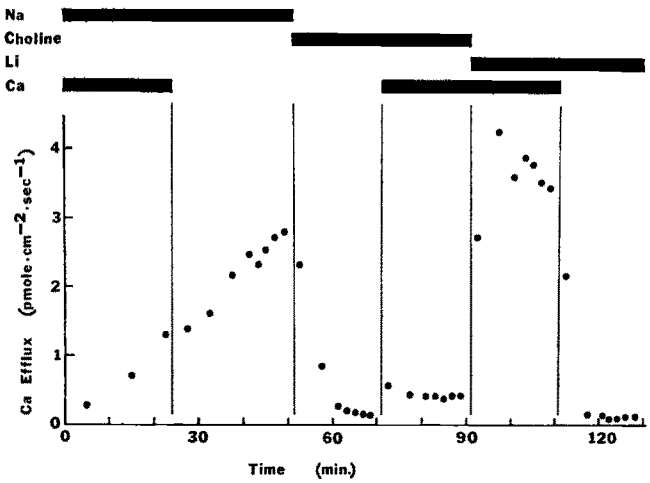


Fig. 7. Effect of external cations on the ^{45}Ca efflux from an internally-dialized squid axon. The bars at the top of the figure indicate the presence of the appropriate cations. The Ca concentration in the Ca-containing solutions was 10 mM; Ca was replaced by Mg in Ca-free solutions. The concentrations of Na, Li and choline were 425 mM. The axon was dialized with an ATP-free solution containing 2 mM cyanide, 50 mM Na and a pCa of 3.3. Axon 6033. Diam. 560 μ. Membrane potential, −56 mV. Temp. 15° C (BLAUSTEIN et al., 1974).

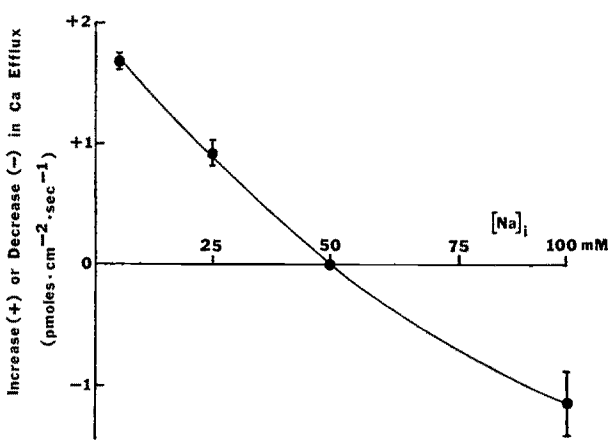


Fig. 8. Effect of internal sodium concentration on ^{45}Ca efflux from internally-dialized squid axons into Ca-free, Na-containing sea water. The graph shows the increment (+) or decrement (−) in the efflux (in pmoles · cm^{-2} · sec^{-1}), relative to the efflux from axons dialized with a solution containing 50 mM Na. For this case ($[Na]_i = 50$ mM), the mean efflux into Ca-free Na-containing sea water was 3.01 ± 0.38 pmoles · cm^{-2} · sec^{-1}. The data are the mean values for 8 axons; the standard errors for the effluxes are indicated by the vertical bars. The axons were dialized with ATP-free solutions containing 2 mM cyanide and a pCa of 3.5–3.7. The sum of $[Na]_i$ and $[K]_i$ (present as the isethionates) was always 400 mM. Taurine (200 mM) accounted for most of the remaining osmotically-active solute in the dialysis fluids. Temp. 15 ± 1° C (BLAUSTEIN et al., 1974).

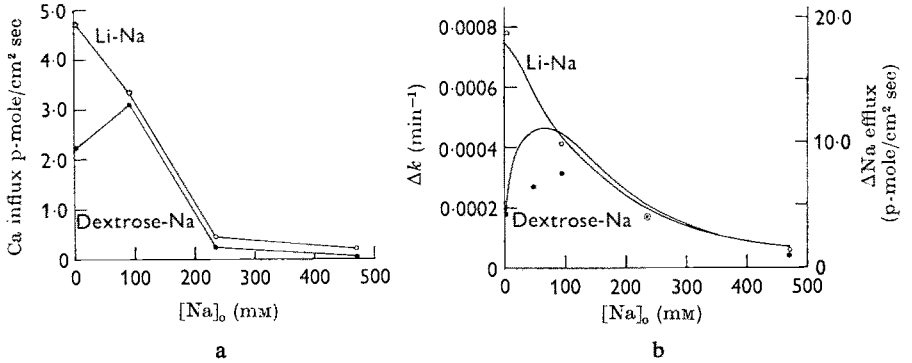


Fig. 9a. Effect of external sodium concentration on calcium influx in squid axons using lithium, upper curve (○), or dextrose, lower curve (●) as replacement for Na. Average results obtained on fourteen axons from seven squid (Li), or sixteen axons from eight squid (dextrose). Abscissa: sodium concentration in mM. Ordinate: calcium influx. K-free solutions with 10^{-5} M ouabain were used

Fig. 9b. Effect of replacing external Na by Li or dextrose on Ca-dependent Na efflux from squid axons in the presence of 10^{-5} M ouabain. ○ Average Ca-dependent sodium efflux for Li–Na mixtures (three experiments). ● Average Ca-dependent sodium efflux for Na-dextrose mixtures (two experiments) in which the efflux in Li sea water was also determined. The mean increment on replacing Na by Li (in the presence of Ca) in these two experiments was the same as in the three experiments from which the Li–Na curve was obtained. Ordinate: left-hand scale, fraction of ^{22}Na lost per minute; right-hand scale, the Na efflux (pmole · cm^{-2} · sec^{-1}) based on an average diameter for these five axons of 822 μ, and a mean intracellular Na concentration for all refrigerated mantle axons tested, of 70 mM/kg axoplasm. The smooth curves were drawn to fit a model described in the original text. (From BAKER et al., 1969a)

discriminate between Na and Li. Thus, reducing Na_o (in the presence of Ca_o) should increase the fraction of the former sites which are occupied by Ca; but the second site must also be occupied – by either Na or Li – in order to permit Ca influx.

A second striking feature of the Ca influx in squid axons is its dependence on Na_i; at low internal Na concentrations (~30 mM), Ca influx increases only slightly when Na_o is replaced by Li, whereas at elevated internal Na concentrations (~130 mM), substitution of Li for Na_o dramatically stimulates Ca influx (BAKER et al., 1969a). The data of Fig. 9a were obtained on axons which presumably had high internal Na concentrations, since the axons came from squid mantles which had been cold-stored for several hours prior to use.

c) External Calcium-Dependent Sodium Efflux

The definitive proof of an ion exchange flux mechanism requires the direct demonstration of appropriately-coupled fluxes moving in opposite directions. Thus, the similarity in the properties of the Ca_o-dependent Ca efflux, and the Ca influx from Na-depleted media, suggests strongly that these are manifestations of Ca–Ca exchange. Direct evidence for Na–Ca exchange in squid axons comes from the observation of a Ca_o-dependent Na efflux (BAKER et al., 1969a), with properties

which bear a strong resemblance to those of the Ca influx: the Na efflux increases when Na_o is progressively replaced by Li or dextrose (or choline); but with dextrose (or choline) as the Na substitute, the Na efflux again declines as $[Na]_o$ is reduced below 100 mM (Fig. 9b). Furthermore, the magnitude of the Li_o-stimulated Na efflux (which is approximately equal to the Ca_o-dependent Na efflux) is directly proportional to the square of the internal Na concentration (BAKER et al., 1969a), and may suggest that two Na^+ ions leave the axon in exchange for each entering Ca^{2+}.

Despite the use of somewhat different techniques for the Ca influx and Na efflux experiments (BAKER et al., 1969a), comparison of the data in Figs. 9a and 9b also provide a crude measure of the Na–Ca exchange stoichiometry – they indicate a Na efflux: Ca influx ratio of 3–5:1. The lower value (3:1), seen at $[Na]_o = 100$ mM, is probably the better estimate, because the nominally "Na-free" solutions used in the Ca influx experiments most likely contained substantial amounts of Na.

Unfortunately, similar data on the Na influx, to help substantiate the Na influx-Ca efflux exchange hypothesis, are not available and may be rather difficult to obtain: the calculated Ca-coupled Na influx, less then 1 pmole/cm^2 sec, would represent (at most) only about one per cent of the total Na influx in the resting squid axon. Nevertheless, despite the absence of direct verification, the circumstantial evidence weighs strongly in favor of the view that a significant fraction of the Ca efflux from squid axons in coupled to Na influx.

d) Do Cardiac Glycosides Directly Affect the Calcium Fluxes?

In most of the aforementioned experiments on the Ca_o-dependent Na efflux, the cardiac glycoside, ouabain, was added to the bathing medium in order to inhibit the K_o-coupled (sodium pump) Na efflux (CALDWELL and KEYNES, 1959) – thereby reducing the Ca_o-independent fraction of the Na efflux. This treatment had no effect on the Ca_o-dependent Na efflux (BAKER et al., 1969a). Even more important, ouabain had no effect on either the Ca efflux (BLAUSTEIN and HODGKIN, 1969) or Ca influx (BAKER et al., 1969a) in squid axons. In a few other preparations, such as crustacean muscle (ASHLEY et al., 1972) and frog skeletal muscle (CURTIS, 1966; see Section IV, A), there also is evidence that cardiac glycosides do not affect Ca fluxes.

This raises a particularly important point because, in a number of the studies to be described in subsequent sections of this review, it will be noted that the application of a cardiac glycoside does, indeed, affect Ca fluxes in a variety of tissues. One possible explanation is that the Ca transport mechanism in these tissues is, itself, directly affected by the glycosides, and thus differs from the mechanism in squid axons. In many instances, however, the removal of external K has been found to mimic the effects of the cardiac glycosides. Consequently, a more likely explanation is that in these tissues the effects of the glycosides on the Ca fluxes are secondary to inhibition of the Na–K coupled pump – and may perhaps result from alterations in the Na and/or K concentration gradients and/or membrane potentials. Such alterations are bound to occur most rapidly in small cells with large surface – to – volume ratios. In large cells, such as squid axons and barnacle

muscle fibers, on the other hand, complete inhibition of the sodium pump should result in only a slow rise in $[Na]_i$ and fall in $[K]_i$, due to the relatively small surface-to-volume ratio.

e) A Model for the Na–Ca Exchange Mechanism in Squid Axons

In the foregoing discussion, the mechanism governing the coupled fluxes of Na and Ca has been referred to by the vague term "transport mechanism". However, a mechanism which can permit exchange diffusion (Ca–Ca exchange), as well as counterflow exchange (Na–Ca exchange), likely involves a mobile carrier (ROSENBERG and WILBRANDT, 1957; WILBRANDT and ROSENBERG, 1961); consequently, the term "carrier" will now be used when referring to the Na–Ca or Ca–Ca transport mechanism.

The evidence presented above suggests that each carrier molecule has two rather distinct sites: one site presumably contains a single negative charge, which confers the voltage-sensitivity on the Ca efflux. In order for the carrier to move inward, this site must be occupied by an alkali metal ion (normally Na^+, although Li^+ or K^+ may also act as the counter-ion). On the outward limb of the cycle, no counter-ion need occupy this site, since the free negative charge would readily be driven down the voltage gradient. [Alternatively, a K^+ could serve as counter-ion on some of the sites – perhaps providing an explanation for the observation that, on the average, somewhat less than one negative charge accompanies the efflux of each Ca ion (see Fig. 6).]

The other type of site on the carrier may, presumably, be occupied by either two Na^+ ions or one Ca^{2+} ion. However, it is difficult to envision a single site which could be so selective for Na, among the alkali metal ions, and also have a very high affinity for Ca^{2+}. A more likely possibility is that this "site" actually has two allosterically different (and mutually exclusive) conformations – one which accepts two Na^+ ions, and another which accepts a single Ca^{2+} ion. For simplicity, we will assume that this carrier "site" has two negative charges, and that the Ca^{2+} or two Na^+ ions act as counter-ions, to provide a net charge of zero at this site. [As an alternative, there may be no net charge at this site on the carrier; instead, the Ca^{2+} (or $2Na^+$) may be liganded to the carrier by means of an induced-dipole interaction as occurs with several ionphorous antibiotics (PRESSMAN, 1968). In this case, anions with a net charge of 2- (for example, two Cl^- ions) may have to accompany the carrier on both its inward and outward journeys. There is, at present, no data available from which to decide between these alternatives.]

The principle features of the proposed carrier model (cf. BLAUSTEIN and HODGKIN, 1969), with the carrier designated as R^{3-}, are depicted in Fig. 10. All of the reactions are presumed to be reversible, but the long arrows indicate the (presumed) preferred directions for each of the individual reactions when a small Ca load is placed on the inside. Under these conditions (a slight displacement from the steady-state), in a single cycle, three Na^+ ions will move down their electrochemical gradient into the axon; one Ca^{2+} ion will exit, moving uphill, along with a negative charge which moves from a negative to a positive potential. Thermodynamic considerations indicate that this carrier could derive sufficient

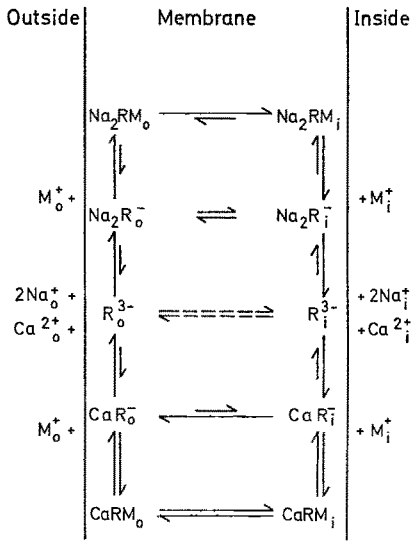


Fig. 10. Model of a sodium-calcium counter-transport carrier mechanism. The free carrier, R^{3-}, is assumed to have two types of cation-binding sites. One site is monovalent, and can bind a single alkali metal ion (M^+); the second site is divalent, with specificity for either Na (two Na^+ ions can bind) or Ca. The chemical reactions between carrier and counter ions are assumed to be very rapid, while the diffusion of the carrier complexes across the membrane are rate-limiting. The diffusible carrier-cation complexes are: Na_2RM, CaRM, Na_2R^- and CaR^- (the latter two are driven by the membrane potential and therefore move preferentially in the outward direction). Free carrier (R^{3-}) is assumed to diffuse slowly, if at all. The long, solid arrows show the directions the reactions would tend to go in if the steady-state were disturbed by a slight increase in $[Ca]_i$. The subscripts, i and o, refer to the axoplasm and hemolymph, or internal and external surfaces of the membrane, respectively

energy from the downhill movements of Na^+ and the free negative charge so that, in the steady-state, the Ca activity gradient (a_{Ca_o}/a_{Ca_i}) would be given by:

$$\frac{a_{Ca_o}}{a_{Ca_i}} = \frac{(a_{Na_o})^3}{(a_{Na_i})^3} \exp \frac{-VF}{RT} \tag{3}$$

Where V is the membrane potential, and R, T and F are the gas constant, absolute temperature, and Faraday's number, respectively. With a Na activity ratio (a_{Na_o}/a_{Na_i}) of about 10, and a -60 mV resting potential, this mechanism could, in principle, provide a Ca activity ratio of about 10^4, thereby holding a_{Ca_i} in squid axoplasm at about 9.3×10^{-8} M (cf. Table 1). This is equivalent to a pCa of about 6.6 which is very close to the value of 6.4–6.5 given by BAKER et al. (1971) and by KATZ and MILEDI (1967), and thus within the likely physiological range.

The apparently-satisfactory fit of this model to much of the available data does not, however, prove that ATP has no direct effect on Na–Ca coupled transport (cf. Section III, A, 1a, and BAKER and GLITSCH, 1973). Moreover, the model does not account for the significant Na_o- and Ca_o-independent Ca efflux, which is particularly prominent in axons with a high (physiological) pCa. The external

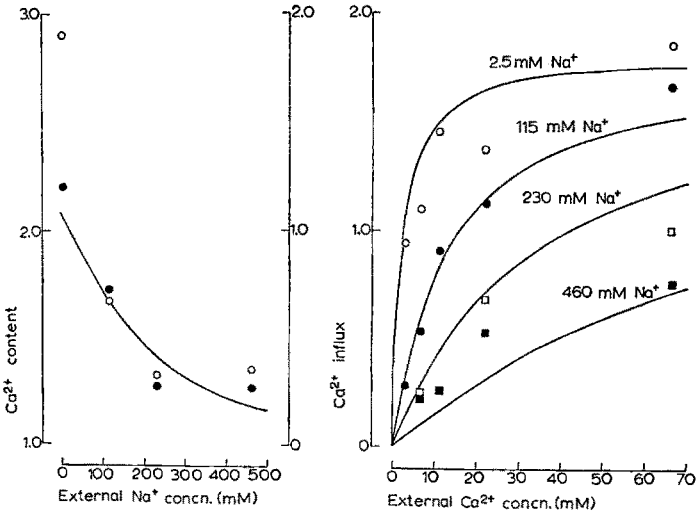


Fig. 11a. Ca^{2+} content (○) (mmoles/kg nerve after 10 min) and Ca^{2+} influx (●) (mmoles/kg nerve during a 7-min exposure to ^{45}Ca) in crab (*Maia*) nerve as a function of the external Na^+ concentration (mM). Na^+-ASW (Na artificial sea water) was replaced isosmotically by Li^+-ASW. The net Ca influx content and Ca^{2+} influx data were obtained on different crabs. Each point is the mean of 3 determinations. An essentially similar curve was obtained when Na^+-ASW was replaced by dextrose-ASW

Fig. 11b. Ca^{2+} influx (mmoles/kg nerve during a 7-min exposure to ^{45}Ca) as a function of the external Ca^{2+} concentration (mM). The solutions were Mg^{2+}-free and Na^+ was replaced is osmotically by Li^+. Each point is the mean of at least 3 separate determinations. The Ca influx (v) curves in Figs. 11a and 11b have been drawn to fit the equation:

$$v = \frac{V}{1 + \frac{K_{Ca}[Mg]_o}{K_{Mg}[Ca]_o} + \frac{K_{Ca}}{[Ca]_o}\left(1 + \frac{[Na]_o}{\bar{K}_{Na}}\right)^2}$$

where V, the maximum rate of Ca influx, is 0.2 M mmoles Ca/kg nerve per min; K_{Ca} and K_{Mg} are the apparent dissociation constants for Ca, Mg and Na, with values of 2 and 20 mM, respectively. $\bar{K}_{Na}$ is the half-saturation constant for Na with a value of 75 mM. (From BAKER and BLAUSTEIN, 1968)

cation-independent efflux could be the manifestation of a second (perhaps ATP-driven) Ca transport system operating in parallel with, and presumably independently of, the Na–Ca exchange carrier. But, despite the precedent in human red blood cells of an ATP-fueled Ca extrusion (SCHATZMANN and VINCENZI, 1969), there is no direct evidence to implicate a similar mechanism in the squid axon or in any cell other than the erythrocyte (although metabolic poison-inhibited Ca extrusion may be circumstantial evidence for such a mechanism; e.g. BORLE, 1969). Furthermore, the operation of two transport systems in parallel, when one may be sufficient to do the job, seems redundant and wasteful. Clearly, additional experimentation is needed to clarify these unresolved problems and to amplify and refine the model or discredit it.

2. Nerves of Other Invertebrates

Although multicellular, small-cell preparations are disadvantageous for certain types of ion flux studies, they may be particularly well-suited for measurement of net ion movements and for the study of some kinetic parameters which require the alteration of several variables in a single experiment. An example of this utility is seen in the results of experiments on the walking leg nerve of the crab, *Maia squinado* (BAKER and BLAUSTEIN, 1968). Bundles of crab nerve fibers (and lobster nerve fibers; BLAUSTEIN and WIESMANN, 1970), pre-loaded with Na by stimulation, accumulated ^{45}Ca much more rapidly from Na-free sea water (Na replaced by Li or dextrose) than from standard Na sea water. The relationship between Ca influx and the external Na concentration is illustrated in Fig. 11a which also shows that there is a net gain of Ca by the fibers incubated in Na-depleted media; the striking similarity between these data and the Ca influx curve for squid axon (Fig. 9a) is obvious. When both Ca_o and Na_o were varied in the same experiment, the results suggested that Na_o was a competitive inhibitor of Ca uptake (Fig. 11b), and that at least two Na^+ ions competed with each Ca^{2+} ion. Accumulation of ^{45}Ca by crab nerve was also influenced by Na_i: uptake from Na-free Li sea water was enhanced in Na-loaded fiber bundles, and inhibited in Na-depleted nerves, as compared to uptake by unstimulated controls. Thus, as in squid, lowering $[Na]_o$ and/or raising $[Na]_i$ tended to move Ca into the tissue.

B. Vertebrate Nervous Tissue

1. Peripheral Nerve

Evidence for Na–Ca exchange in vertebrate peripheral nerve axons is limited to the demonstration that the ^{45}Ca efflux from rabbit vagus was inhibited when external Na was replaced by choline (KALIX, 1971); however, the inhibitory effect was only "slight" when Li was substituted for Na. Neither 10^{-4} M ouabain, which inhibits K transport in C fibers (KEYNES and RITCHIE, 1965), nor the metabolic poisons, cyanide, monoiodoacetate and 2,4-dinitrophenol had any effect on the Ca efflux.

Indirect evidence regarding Na–Ca exchange at vertebrate motor nerve terminals will be discussed in Section III, B, 2b.

2. Brain

a) Brain Slices

The mammalian brain slice has been a much more popular preparation for studying Ca transport than peripheral nerve, and a number of these slice studies suggest the occurrence of Na–Ca exchange processes in the mammalian central nervous system. There is, first of all, a marked increase in Ca accumulation when external Na is replaced by Li, choline or sucrose. The shape of the curve relating Ca uptake to $[Na]_o$ is very similar to the one obtained with invertebrate nerve

preparations (cf. Figs. 9a and 11a), and little stimulation of Ca uptake is observed unless more than half the external Na is removed (COOKE and ROBINSON, 1971; STAHL and SWANSON, 1972). Ca uptake is also enhanced by conditions which inhibit the Na–K coupled pump, causing the loss of K and gain of Na by cerebral tissues; these conditions include incubation with ouabain or with metabolic poisons (cyanide, azide or 2,4-dinitrophenol), incubation with K-free media, and anoxia (FUJISAWA et al., 1965; TOWER, 1968; STAHL and SWANSON, 1969 and 1972; COOKE and ROBINSON, 1971; BULL and TREVOR, 1972). The fact that ouabain and K-free solutions are effective indicates that the extra Ca accumulation is probably not a direct consequence of inhibited ATP production; this leaves depolarization and increased $[Na]_i$ or decreased $[K]_i$ as possible stimulators of Ca uptake.

Most of the ouabain-induced Ca accumulation was found in the mitochondrial fraction of the tissue homogenates (TOWER, 1968; STAHL and SWANSON, 1969; COOKE and ROBINSON, 1971), but the rate of Ca accumulation by isolated brain mitochondria was not influenced by ouabain (STAHL and SWANSON, 1969) – which does not conflict with the view that the only direct cellular action of cardiac glycosides is inhibition of the plasma membrane Na–K coupled pump (*cf.* LEE and KLAUS, 1971, and Section IX, A).

Ca efflux from ^{45}Ca-loaded brain slices is also influenced by Na_o, and perhaps by Na_i. Tracer efflux into Ca-free (STAHL and SWANSON, 1972) and Ca-containing (COOKE and ROBINSON, 1971; BULL and TREVOR, 1972) media is reduced when external Na is replaced by choline or sucrose, or when ouabain is added to the medium. As in squid axons (Section III, A, 1a), azide (FUJISAWA et al., 1965) and cyanide (STAHL and SWANSON, 1972), which should reduce pCa, accelerate the efflux of ^{45}Ca into Ca-free solutions.

The foregoing results show clearly that Ca accumulation is enhanced when the $[Na]_o/[Na]_i$ gradient is reduced; but lowering the gradient with Na-depleted external media or with ouabain inhibits Ca efflux, whereas metabolic poisoning stimulates Ca efflux. It should be recognized, however, that the influence of some of these treatments is difficult to interpret. In small cells (i.e., those with a large surface: volume ratio), inhibitors of the Na–K pump and inhibitors of intermediary metabolism not only cause $[Na]_i$ to increase rapidly; they also cause a reduction of $[K]_i$ and concomitant depolarization – and, as seen in the squid axon, depolarization may directly influence Ca efflux.

A further complication of the brain slice preparation is that it contains a considerable number of non-nervous elements, primarily glia. Consequently, there is uncertainty as to which cells in the brain slice are responsible for the Na-dependent Ca fluxes.

b) Presynaptic Nerve Terminals

Studies on pinched-off presynaptic nerve terminals (synaptosomes) separated from mammalian brain homogenates may help to resolve the aforementioned dilemma, since this preparation is composed almost exclusively of neural elements. Data from many laboratories (see review by RODRIGUEZ DE LORES ARNAIZ and DE ROBERTIS, 1972) indicate that these pinched-off terminals reseal and retain many

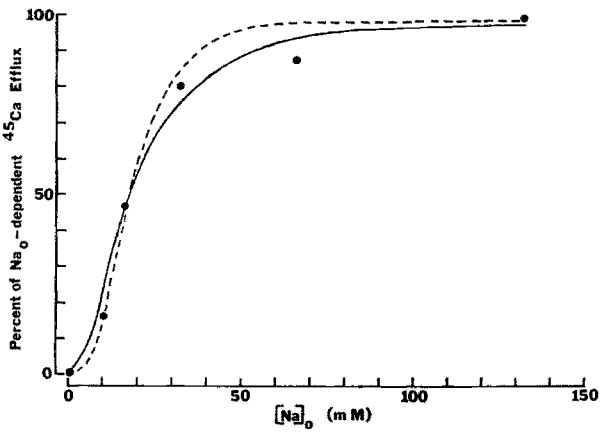
of the physiological activities normally associated with intact cells; they have complete glycolytic and oxidative pathways, and can utilize metabolic energy to accumulate and retain K, and to extrude Na. They likely have membrane potentials (GOLDRING and BLAUSTEIN, 1973) and can release transmitters when "depolarized" (e.g. BLAUSTEIN et al., 1972). There is now considerable evidence that transmitter release is normally triggered by the entry of Ca at the nerve terminals (KATZ, 1969; KATZ and MILEDI, 1970; BLAUSTEIN, 1971; LLINAS et al., 1972; BLAUSTEIN et al., 1972). The functional significance of an extrusion mechanism for Ca at nerve terminals is thus readily apparent.

At the neuromuscular junction, the frequency of miniature end-plate potentials (m.e.p.p.) has been used as an index of transmitter release, and, indirectly, as a measure of pCa in the motor nerve terminals (*cf.* Section II, B). Thus, the increased m.e.p.p. frequency which results from a reduction of $[Na]_o$, as a consequence of Na–Ca competition (BIRKS and COHEN, 1965; KELLY, 1965; GAGE and QUASTEL, 1966), or a rise in $[Na]_i$ (BIRKS and COHEN, 1965 and 1968a and b; MUCHNIK and VENOSA, 1969) may indicate that these conditions tend to lower pCa by driving Ca into the terminals on an Na–Ca exchange carrier (BIRKS and COHEN, 1968b).

The release of norepinephrine from sympathetic nerve endings in the cat spleen is also stimulated by Na-deprivation (KIRPEKAR and WAKADE, 1968), and by ouabain if Ca is present (GARCIA and KIRPEKAR, 1973). Similarly, Na-depletion enhances the Ca_o-dependent release of norepinephrine from rat heart (BLASZKOWSKI and BOGDANSKI, 1971).

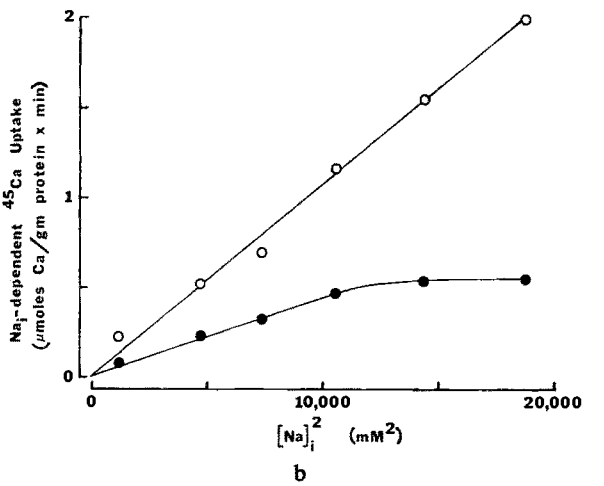
Of particular interest, in the context of the present article, is the observation that the isolated nerve terminals (synaptosomes) exhibit Ca fluxes which require the presence of Na on the side of the membrane toward which Ca moves (BLAUSTEIN and WIESMANN, 1970; BLAUSTEIN and OBORN, 1974). Quantitative assessment of this Na dependency indicates that the Ca efflux may be directly related to $[Na]_o^3$ (Fig. 12a, and compare Figs. 4 and 14). The relationship between $[Na]_i$ and Ca influx is rather complex, and is in part dependent upon $[Na]_o$ as well. Ca uptake from Na-free Li medium containing 1.2 mM Ca is proportional to $[Na]_i^2$; Uptake from the standard Na-containing medium is also proportional to $[Na]_i^2$, but appears to saturate at high $[Na]_i$ (Fig. 12b and see Fig. 13b). When $[Ca]_o$ is increased to 7.6 mM, Na_i-dependent Ca uptake from the Na-containing medium does not saturate as $[Na]_i$ is increased to 137 mM (BLAUSTEIN and OBORN, 1974). These data may indicate that at a low $[Na]_i$, and with $[Na]_o = 137$ mM and $[Ca]_o = 1.2$ mM, the rate of carrier movement from inside to outside (presumably as Na_2R^- or Na_3R; *cf.* Fig. 10) is the rate-limiting step in the Na–Ca exchange cycle. However, as $[Na]_i$ is increased, and the carrier cycles more rapidly, the binding of Ca_o to the carrier may become rate-limiting because of Na_o–Ca_o competition.

Ca influx is also inhibited by external Na, and the curve relating Na_i-dependent Ca uptake to $[Na]_o$ (BLAUSTEIN and WIESMANN, 1970) is comparable to the curves obtained with crab nerve (Fig. 11a), squid axon (Fig. 9a) and mammalian brain slices (e.g. STAHL and SWANSON, 1972). When the effects of both $[Na]_o$ and $[Ca]_o$ were tested, a family of curves very similar to those of Fig. 11b were obtained (BLAUSTEIN and OBORN, 1974), perhaps indicating that, as in crab nerve, at least two Na^+ ions compete with each Ca^{2+} for entry.



a

Fig. 12a. External sodium-dependent Ca efflux from ^{45}Ca-loaded rat brain synaptosomes graphed as a function of $[Na]_o$. Choline was substituted for Na_o, and the sum of Na+choline was always 132 mM; all of the efflux solutions were Ca-free. The Na_o-dependent ^{45}Ca efflux into Ca-free Na solution, 0.29 μmoles Ca/mg protein per min during a two-minute incubation at 30° C, was taken as 100 per cent. The ^{45}Ca efflux into the Na-free, Ca-free choline solution averaged 0.03 μmoles/mg protein per min. Each point is the mean of four determinations. The smooth curves were drawn to fit the equation shown in the caption to Fig. 4. In this case, $\overline{K}_{Na}$ had a value of 18 mM. The exponent, n, had a value of 2 (solid curve) or 3 (broken curve)



b

Fig. 12b. Internal sodium-dependent ^{45}Ca influx in synaptosomes, graphed as a function of $[Na]_i^2$. The rat brain synaptosomes were first equilibrated with Ca-free solutions containing mixtures of Na and Li, with the sum of Na+Li always equal to 137 mM. Ouabain (10^{-3} M) was also present, to minimize Na extrusion from the Na-loaded synaptosomes. The equilibration solution was decanted following centrifugation, and the pellets were re-suspended in a medium containing either 137 mM Li (○) or 137 mM Na (●). Synaptosomes were incubated in these solutions, which also contained 1.2 mM Ca labeled with ^{45}Ca, for 1 min at 30° C. Uptake of ^{45}Ca was terminated by the addition of EGTA, and the extra-synaptosomal ^{45}Ca was removed as described by BLAUSTEIN and WIESMANN (1970). The Na concentration graphed on the abscissa is the Na concentration in the equilibration solution, and therefore, presumably, the concentration inside the synaptosomes (BLAUSTEIN and OBORN, unpublished data)

Metabolic poisons also affect the Ca fluxes in synaptosomes: cyanide inhibits Ca influx and enhances Ca efflux into Ca-free media. Cyanide and 2,4-dinitrophenol both cause a net loss of Ca from terminals incubated in media which contain 1.2 mM Ca (BLAUSTEIN and OBORN, 1974).

The main conclusion from these experiments is that an Na–Ca exchange mechanism, with many properties similar to those observed in squid axons, probably is present in presynaptic nerve endings. As in squid axons, the exchange mechanism can extrude Ca from metabolically-poisoned preparations; this may indicate that ATP is not necessarily the immediate source of energy for this process.

IV. The Influence of Sodium on Calcium Fluxes in Muscle

In many types of muscle, including invertebrate muscle and vertebrate cardiac and smooth muscle, Ca^{2+} ions may carry a considerable fraction of the inward current during depolarization – and, particularly in those tissues where the sarcoplasmic reticulum appears to be rather sparse, this "extra" entry of Ca during activity may be essential for the activation of contractile proteins (*cf.* REUTER, 1973). However, even in vertebrate (frog) skeletal muscle, which can contract perfectly well in the absence of external Ca (ARMSTRONG et al., 1972) there is normally an increased entry of ^{45}Ca during activity (BIANCHI and SHANES, 1959; CURTIS, 1966; and see BIANCHI, 1968). Virtually all types of muscle fibers must, therefore, be able to extrude Ca against an electrochemical gradient in order to return to the steady-state following a period of activity.

In muscle, contraction, as indicated by tension development or shortening, may be used to "bioassay" changes in pCa (*cf.* FILO et al., 1965; HELLAM and PODOLSKY, 1969), and perhaps indirectly, changes in Ca fluxes. When contracture occurs following an alteration of external cation concentration, there may be reason to suspect that the immediate cause of the contracture is a fall in pCa as a result of increased Ca influx and/or decreased Ca efflux – provided that release of Ca from the sarcoplasmic reticulum can be ruled out. The absolute requirement for Ca_o, in the absence of membrane potential changes (to avoid voltage-dependent changes in Ca conductance and Ca release from sarcoplasmic reticulum), may provide presumptive evidence that the contracture is a consequence of net inward movement of Ca.

A. Vertebrate Skeletal Muscle

Information regarding Ca transport mechanisms in vertebrate skeletal muscle is rather limited, but the study by COSMOS and HARRIS (1961) strongly suggests that Na–Ca exchange may occur in frog muscle. They observed a close correlation between the net gain of Na and of Ca. Conditions which promoted Na gain and K loss also promoted ^{45}Ca and net Ca entry: incubation in K-free media, in ice-

cold media, or in media containing ouabain. Net re-extrusion of Ca (and Na) was observed when tissue stored in a cold, K-free environment was returned to Ringer's containing 10 mM K. Ca influx (using strontium-89 as a tracer, since the movements of ^{45}Ca and ^{89}Sr seemed comparable) and net Ca gain were also enhanced by depletion of Na_o. In one experiment, a reduction of $[Na]_o$ from 100 to 30 mM increased the rate of ^{89}Sr uptake, and a return to the 100 mM Na medium induced net extrusion of the isotope despite its continued presence in the external medium. These observations led COSMOS and HARRIS to conclude that the Ca entry mechanism probably involved Na–Ca competition for anionic sites which could accept either cation. There is also evidence for Na_o–Ca_o competition in slow muscle fibers, since reduction of $[Na]_o$ induces sustained contractures in the frog *rectus abdominis* (SCHAECHTELIN, 1961).

CURTIS (1966), studying ^{45}Ca fluxes in single frog muscle fibers, noted that cardiac glycosides had no effect on Ca influx or on the time constant for Ca efflux. Since the incubation period used by CURTIS was much shorter than that used by COSMOS and HARRIS, and since the rate of net Na gain by glycoside-poisoned frog muscle is relatively slow (EDWARDS and HARRIS, 1957), these observations indicate that, as in nerve, cardiac glycosides have no direct effect on the Ca fluxes. Rather, their effects on Ca fluxes (in the experiments of COSMOS and HARRIS) are probably a consequence of the slow increase in $[Na]_i$.

B. Cardiac Muscle

The earliest evidence of an antagonism between Na_o and Ca_o was WILBRANDT and KOLLER's (1948) demonstration that the strength of contraction of the frog heart is directly related to the ratio, $[Ca]_o/[Na]_o^2$. These observations were confirmed and extended by LÜTTGAU and NIEDERGERKE (1958; and see NIEDERGERKE and LÜTTGAU, 1957), who showed that even in depolarized cardiac muscle, contraction could be effected by depleting the medium of Na. The contractures in Na-poor media could be accounted for by NIEDERGERKE's (1963, and see NIEDERGERKE, 1954; NIEDERGERKE and HARRIS, 1956) observation that a marked increase in Ca influx and net gain of Ca occurred in frog ventricles exposed to the contracture fluids, in which Li, choline or dextrose was substituted for Na. Prompt relaxation occurred (see also VASSORT, 1973) in conjunction with a net loss of Ca upon return of the Ca-loaded ventricles to control Ringer's. To explain these results, NIEDERGERKE postulated that Ca entry occurred via a carrier mechanism for which external Na^+ and Ca^{2+} ions competed, presumably in the ratio, 2:1.

REUTER and his colleagues (REUTER and SEITZ, 1968; GLITSCH et al., 1970) examined the effects of both internal and external Na on Ca fluxes in mammalian cardiac muscle (guinea pig auricles, and calf and sheep ventricular trabeculae). As in the frog heart, partial or complete replacement of Na_o increased Ca influx and the net Ca content of the tissue. The most dramatic increase was observed in tissue which had been pre-loaded with Na, as shown in Fig. 13, where Ca uptake from Na and from Li media is graphed as a function of $[Na]_i^2$. The striking similarity between these data and the data from synaptosomes (Fig. 12b), including

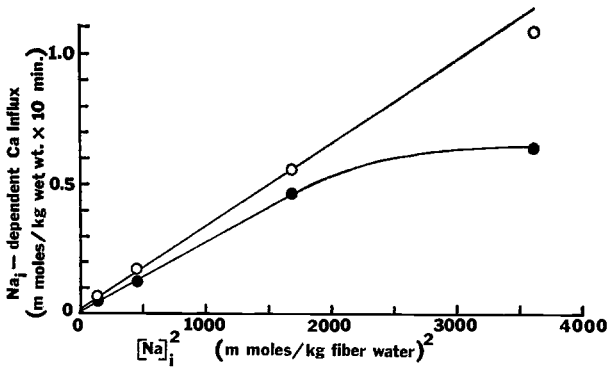


Fig. 13. Internal sodium-dependent ^{45}Ca influx in guinea pig auricles (data of GLITSCH et al., 1970, Fig. 3) graphed as a function of $[Na]_i^2$. The auricles were incubated for 10 min in ^{45}Ca-containing Tyrodes solution (●) or in a similar solution with 137 mM NaCl replaced by choline Cl (○)

the apparent saturation of Ca uptake under conditions of high $[Na]_i$ and high $[Na]_o$ (cf. Section 1B, 2b), is readily apparent.

The Ca efflux from mammalian heart (REUTER and SEITZ, 1968) was dependent upon both Na_o and Ca_o: it was reduced to 70 per cent of control levels when Ca_o was removed, and to 20 per cent of control levels when Na_o was also removed (replaced by Li, choline or sucrose). The Ca_o-dependent fraction of the Ca efflux was inhibited by Na_o, but was constant at a constant $[Ca]_o/[Na]_o^2$ ratio, suggesting that Ca_o and Na_o compete for external binding sites on a Ca carrier in the ratio, 2:1.

All of these observations in cardiac muscle are consistent with a Na–Ca exchange carrier mechanism. However, they only provide evidence for a 2 Na^+-for-1 Ca^{2+} exchange, which would clearly be insufficient to maintain the observed Ca electrochemical gradient. Obviously, more complex Ca transport mechanisms, such as the 3-for-1 model of Fig. 10 or perhaps an ATP-dependent system, must be considered, although no information regarding these possibilities is yet available.

C. Smooth Muscle

The situation *vis-a-vis* the possible role of Na–Ca exchange in smooth muscle is complicated by the fact that many types of smooth muscle undergo spontaneous electrical discharge. Since the depolarizing current may be carried by Ca^{2+} and/or Na^+ ions (*cf.* KURIYAMA, 1970; REUTER, 1973; TOMITA and WATANABE, 1973), alterations in the extracellular or intracellular concentrations of these ions might affect tension by mechanisms unrelated to a carrier-mediated transfer of Ca. Nevertheless, some observations on the effects of changes in $[Na]_o$ and $[Na]_i$ on smooth muscle coincide fairly closely with the pattern suggestive of Na–Ca exchange in other tissues. For example, JUDAH and WILLOUGHBY (1964) found that segments of guinea-pig ileum go into contracture when exposed to Na-free media (Li or sucrose substituted for Na); relaxation requires the presence of Na in the

medium, and is incomplete when ouabain is also present. Sustained contractures are also induced in mouse myometrium by Na-depleted solutions (OSA, 1971). Although taenia coli do not give sustained contractures in Na-free media, the contractures induced by K-rich media are maintained if the tissue is then transfered to media containing sucrose or tris-hydroxymethylamino methane in place of Na; but slow relaxation does occur when Li is substituted for Na (KATASE and TOMITA, 1972; and see TOMITA and WATANABE, 1973). The rate of relaxation in Na-containing media is slowed if ouabain is added to, or if K is deleted from the medium (KATASE and TOMITA, 1972). These observations fit with the idea that Ca extrusion by taenia coli is dependent upon a large Na gradient in the normal direction. The fact that the Ca content of taenia coli is increased upon exposure of the tissue to Na-free sucrose (GOODFORD, 1967), or to K-free or ouabain-containing media (CASTEELS et al., 1973), supports this view.

In rat portal vein, the rate of relaxation following K-induced contractures is also slowed by the deletion of external Na, although incubation in an Na-free medium does not, itself, induce contracture (BIAMINO and JOHANSSON, 1970).

A somewhat more complete and straightforward picture is available for arterial smooth muscle: removal of external Na induces contractures (e.g. HINKE and WILSON, 1962; SITRIN and BOHR, 1971; and see the reviews by BOHR, 1964, and SOMLYO and SOMLYO, 1968) without depolarization (REUTER et al., 1973). The contracture tension is influenced by both Ca_o and Na_o and, as in the heart (Section IV, B), is approximately constant at a constant $[Ca]_o/[Na]_o^2$ ratio (REUTER et al. 1973). There is some indication that increasing $[Na]_i$ may also affect tension in arterial smooth muscle, since incubation in K-free media or in media containing ouabain causes contractures (LEONARD, 1956) despite only a 5–10 mV depolarization (REUTER et al., 1973). The contractures which occur in Na-depleted media are associated with a net gain of Ca by the tissue (REUTER et al., 1973) as a result of both increased Ca influx (VAN BREEMEN and MCNAUGHTON, 1970; VAN BREEMEN et al., 1973) and decreased Ca efflux (REUTER et al., 1973).

In sum, arterial smooth muscle, and perhaps other types of smooth muscle, have many of the properties which indicate that Ca transport in these tissues may involve Na–Ca exchange. Furthermore, this mechanism may play a role in the maintenance of resting tension in arterial muscle which apparently has a relatively sparse sarcoplasmic reticulum (DEVINE et al., 1972) with a less active Ca pump than skeletal muscle (FITZPATRICK et al., 1972): In normal man (e.g. MASON and BRAUNWALD, 1964) and dog (HIGGINS et al., 1972), pharmacological doses of cardiac glycosides induce vasoconstriction by a direct action on the vasculature. These observations may indicate that even a small increase in $[Na]_i$, due to partial inhibition of the Na–K pump by the cardiac glycosides, may tend to move Ca into the smooth muscle cells thereby lowering pCa and concomitantly increasing resting tension.

D. Invertebrate Muscle

The muscle fibers of certain crustaceans, most notably those of the giant barnacle, *Balanus nubilus*, are sufficiently large (diameters up to 2–3 mm) to permit the use

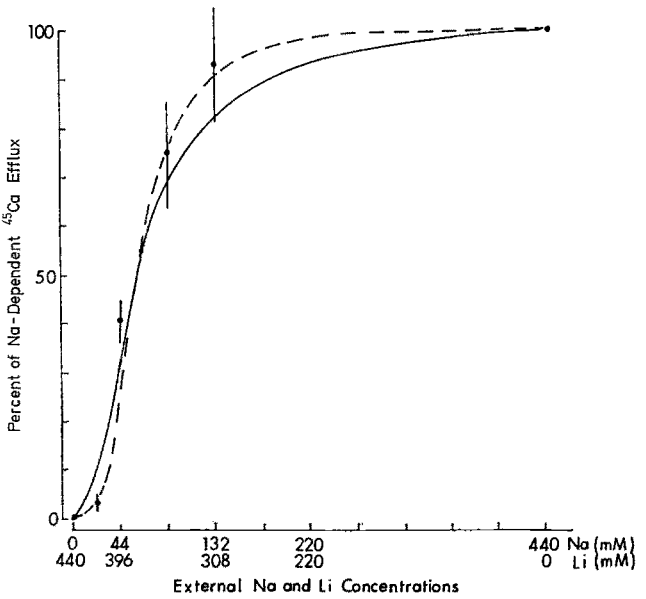


Fig. 14. Per cent of the Na_o-dependent ^{45}Ca efflux from ^{45}Ca-injected barnacle muscle fibers in Ca-free sea waters graphed as a function of the external Na and Li concentrations; the Na_o-dependent Ca efflux into Ca-free sodium-containing sea water was taken as 100 per cent. The graphed points are the mean values for three experiments; the vertical bars through the points indicate $\pm$ one standard error. The experiments were conducted at temperatures of $18 \pm 1°$ C. The data have been fitted to the equation shown in the caption to Fig. 4; in this case $\overline{K}_{Na}$ had a value of 60 mM. The exponent, n, had a value of 2 (solid curve) or 3 (broken curve). In these experiments the mean Na_o-dependent Ca efflux in Ca-free Na-containing sea water was about 0.6 pmoles $\cdot$ cm^{-2} $\cdot$ sec^{-1}, assuming complete mixing of the isotope in the sarcoplasm. (From RUSSELL and BLAUSTEIN, 1974)

of intracellular injection (*cf.* CALDWELL and WALSTER, 1963) and internal dialysis (*cf.* BRINLEY and MULLINS, 1967) techniques for ion flux measurements. This situation has been used to advantage, and Ca flux studies on muscle fibers of *Balanus nubilus* have been carried out recently in several laboratories. Ca influx in intact fibers (BLAUSTEIN and RUSSELL, 1974) and in internally-dialyzed fibers (DIPOLO, 1973b) is stimulated when $[Na]_o$ is decreased and/or $[Na]_i$ is increased. In the absence of external Na, and with $[Na]_i$ increased there is a large net gain of Ca and contracture. BRINLEY (1968) observed that under these conditions the contracture was associated with a dramatic increase in Na efflux. This Na efflux appears to be Ca_o-dependent, thereby providing direct evidence for Na–Ca exchange in barnacle muscle (BLAUSTEIN and RUSSELL, 1974).

Studies on the effects of $[Ca]_o$ and $[Na]_o$ on shortening velocity may provide a crude indication of the interactions between these cations and the Ca "carrier": Na competitively inhibits the stimulatory action of Ca on shortening velocity, where two Na^+ ions appear to compete with one Ca^{2+} (BLAUSTEIN and RUSSELL, 1974).

Ca efflux from *Balanus* muscle is also influenced by internal and external cation concentrations. In intact fibers injected with ^{45}Ca, the rate of tracer loss is reduced by about 50 per cent when Ca_o is removed; of the remaining Ca efflux into Ca-free media, a variable fraction averaging about 40 per cent is Na_o-dependent (RUSSELL and BLAUSTEIN, 1974). The magnitude of the Na_o-dependent fraction could be increased at the expense of the Ca_o- and Na_o-independent portion of the efflux, by treatment with 2–3.5 mM caffeine (which should release Ca from the sarcoplasmic reticulum; *cf.* WEBER and HERZ, 1968). This observation suggests that a slight fall in pCa (but insufficient to induce observable contracture in this case) may perhaps stimulate Na–Ca exchange (*cf.* RUSSELL and BLAUSTEIN, 1974). The quantitative relationship between $[Na]_o$ and the Na_o-dependent Ca efflux is shown in Fig. 14; the fact that the sigmoid curve fits best to a cubic equation raises the possibility that, as in the squid axon (Section III, A, 1), one exiting Ca^{2+} may exchange for 3 Na^+ ions (*cf.* Fig. 10).

There is also evidence for competition between Na and Ca at the internal surface of the membrane, since Ca efflux from internally-dialyzed fibers is increased by 50 per cent when $[Na]_i$ is reduced from 80 to 2 mM (VOGEL and BRINLEY, 1973).

Although many details remain to be filled in, these data suggest that the Na–Ca exchange mechanisms in barnacle muscle fibers and in squid axons may be quite similar. In crayfish muscle, where relaxation from caffeine-induced contractures is significantly slowed in Na-depleted media (ORENTLICHER and ORNSTEIN, 1971) Na–Ca exchange may also play a role in trans-sarcolemmal Ca regulation.

V. Secretory Tissues

A large variety of secretory processes, including the release of neural transmitter substances (see Section III, B, 2b), are critically dependent upon extracellular Ca, and in many instances secretion has been correlated with the entry of Ca into secretory cells (DOUGLAS, 1968; RUBIN, 1970). Although the mechanism of "stimulus-secretion coupling" (DOUGLAS and RUBIN, 1961; and see DOUGLAS, 1968) is incompletely understood, if secretion is indeed triggered by Ca entry and decreased pCa (*cf.* DOUGLAS, 1968; KATZ, 1969), secretory activity may serve as a useful, albeit crude measure of decreased pCa (*cf.* LLINAS et al., 1972). Considerable evidence has accumulated from this type of "bioassay" to implicate Na–Ca exchange as one mechanism of Ca entry in a variety of secretory tissues. This view is based upon observations that Na_o-deprivation, and treatments which may be expected to inhibit the Na–K coupled pump and increase $[Na]_i$, stimulate secretion.

The influence of Na on catecholamine release at adrenergic synapses has already been mentioned (Section III, B, 2b), and a very similar situation appears to apply in the case of catecholamine release from the adrenal medulla: the basal secretion (i.e. in the absence of a secretagogue) of catecholamines is enhanced when external Na is replaced by sucrose or Li (DOUGLAS and RUBIN, 1961 and 1963; BANKS et al., 1969). This effect is Ca_o-dependent (DOUGLAS and RUBIN, 1961 and 1963), and is not a consequence of depolarization—in fact it is accompanied by a

slight hyperpolarization of adrenal medullary cells (DOUGLAS et al., 1967b). Perfusion of adrenal glands with K-free media (DOUGLAS and RUBIN, 1961; BANKS et al., 1969) or with media containing ouabain (BANKS, 1967) also enhances basal secretion, and BANKS (1967) has shown that the ouabain effect is Ca_o-dependent. Metabolic inhibition by anoxia and glucose-deprivation, which should also increase $[Na]_i$, potentiates Ca_o-dependent spontaneous catecholamine release (RUBIN, 1969).

The basal secretion of insulin by the β-cells of the pancreas is likewise stimulated by Na_o-deprivation (with replacement by Li or choline), by K_o-deprivation, and by ouabain (HALES and MILNER, 1968a). The stimulatory effect of ouabain is abolished in the absence of either Ca_o or Na_o (HALES and MILNER, 1968a and b). Insulin release from cultured fetal rat pancreas also is stimulated by K-free media, if Ca_o and Na_o are both present, and by Na_o-depletion (LAMBERT et al., 1969). And in the intact dog, infusion of ouabain in the hepatic portal vein induces hypoglycemia as a result of enhanced insulin release (TRINER et al., 1968).

Some secretions of both the anterior and posterior pituitary are enhanced by ouabain: DICKER (1966) observed an increased release of oxytocin and vasipressin from rat neurohypophysis in the presence of cardiac glycoside. In glands from adult rat, but apparently not from newborns, the ouabain effect was Ca_o-dependent although no special precautions were taken to assure complete removal of Ca^{2+} from the Ca-free solutions.

Ouabain also stimulates the Ca_o-dependent secretion of adrenocorticotropin (ACTH) and growth hormone (FLEISCHER et al., 1972), as well as a concomitant accumulation of ^{45}Ca by the adenohypophysis (FLEISCHER and WOOD, 1973). Deprivation of K_o also promotes growth hormone secretion and potentiates the effect of cardiac glycoside on ACTH secretion (FLEISCHER et al., 1972).

One interpretation of the foregoing observations is that reduction or reversal of the Na gradient in these secretory tissues may tend to move Ca into glandular tissues by an Na–Ca exchange mechanism, thereby lowering pCa and triggering the release of secretory product. Clearly, this is not a normal physiological mechanism for triggering secretion. More physiologic secretogogues appear to depolarize secretory cells (*e.g.* DOUGLAS et al., 1967a; DEAN and MATTHEWS, 1968; and see DOUGLAS, 1968 and KATZ, 1969), perhaps inducing a depolarization-dependent increase in Ca permeability and Ca influx (*cf.* DOUGLAS, 1968; KATZ, 1969). Obviously, a Ca extrusion mechanism would then be necessary for recovery, and it seems possible that Na–Ca exchange could participate by operating in the opposite direction from that just described – although there is no experimental evidence available to support this hypothesis.

VI. Intestinal and Renal Epithelia

A. Intestinal Absorption of Calcium

Electrical (ROSE and SCHULTZ, 1971) and Ca concentration gradient (*cf.* Section II, B and CASSIDY et al., 1969) considerations indicate that there is normally a large electrochemical gradient favoring the movement of Ca from the mucosal lumen,

and from serosal fluid, into the mucosal cells. Consequently, looking at Ca absorption from a thermodynamic viewpoint, there appears to be little problem in getting Ca from the intestinal lumen into the mucosal cells, but energy must be expended to extrude Ca across the serosal (lateral-basal) border of these cells. Although the mechanism of this uphill process is not understood, data from two laboratories (MARTIN and DE LUCA, 1969; SCHACHTER, 1969; SCHACHTER et al., 1970) show clearly that Na is required, presumably in the serosal medium (although this was not tested specifically), in order to effect the transport of Ca from cell to serosal fluid. Reduction of the [Na] in the lumen solution, alone, has been reported to have a stimulatory (GIBBONS et al., 1972) or slight inhibitory effect (LAUTERBACK, 1967a) on Ca absorption, and SCHACHTER et al. (1970) found that Na has little effect on the fluxes of Ca across the brush border of the mucosal cells.

Ca absorption is inhibited by cardiac glycosides (LAUTERBACH, 1967b; ADAMS and NORMAN, 1970), which must be present in the serosal medium to be effective (ADAMS and NORMAN, 1970). They have no effect when placed only in the mucosal (lumenal) medium (ADAMS and NORMAN, 1970; GIBBONS et al., 1972). These observations are consistent with other evidence that Na–K coupled pumps are present primarily on the serosal surface of intestinal mucosal cells (SMITH, 1964; CSAKY and HARA, 1965), and that cardiac glycosides act primarily at the pump sites on the external surfaces of cells (CALDWELL and KEYNES, 1959).

A proposal for a model of the Ca absorption process in the intestine which takes the foregoing observations into account is illustrated in Fig. 15; it incorporates many of the ideas suggested by MARTIN and DE LUCA (1969). Ca^{2+} presumably moves (by an Na-independent route) down its electrochemical gradient from lumen to cell, with the rate of transfer being determined by the Ca permeability of the brush border. At the serosal side of the cell a carrier mechanism, perhaps similar to the one illustrated in Fig. 10, exports Ca up its electrochemical gradient in exchange for Na, which moves downhill. Although not shown in the figure, there is evidence for a vitamin D-dependent Ca-binding protein in the mucosa cell cytosol, which might facilitate the movement of Ca from mucosal to serosal border within the cell, while maintaining a high pCa (*cf.* WASSERMAN, 1968).

B. Reabsorption of Calcium by the Kidney

The renal tubules are constantly faced with the task of reabsorbing nearly all of the Ca which is filtered at the glomeruli – about 99 per cent, or approximately 200 millimoles/day in a normal adult man. Micropuncture studies in rodents indicate that Ca is transported out of all major regions of the nephron lumen against an electrochemical gradient, although most of the reabsorption takes place in the proximal convoluted tubule (LASSITER et al., 1963). A significant fraction of the tubular Ca might perhaps be reabsorbed by bulk flow (of water and some solutes) through the paracellular shunt pathways between the renal tubular cells (*cf.* BOULPAEP, 1972; FRÖMTER and DIAMOND, 1972). However, this mechanism alone could not produce a tubular fluid/plasma ratio for dializable Ca of significantly less than 1.0 (*cf.* LASSITER et al., 1963) in the absence of a large, positive

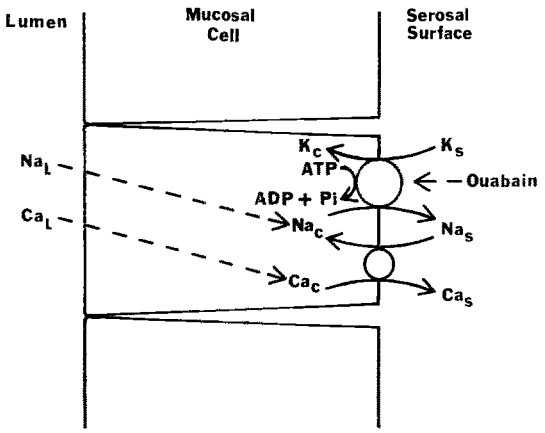


Fig. 15. Model for the mechanism of transport of Ca from intestinal lumen to blood plasma. Calcium is presumed to move passively (possibly by facilitated diffusion) across the brush border from intesinal lumen to mucosal cell cytoplasm. Within the cytosol, the Ca may diffuse from the lumenal to the serosal border—either as the free ion, or perhaps complexed to Ca-binding protein (see text). The uphill transport of Ca across the serosal plasma membrane is then effected by Na–Ca exchange—by a carrier mechanism perhaps similar to the one depicted in Fig. 10. Note that this carrier is assumed to operate in parallel with, and independently of the ATP-dependent Na–K exchange pump. Whether or not ATP is also directly required for Ca transport is unknown. The mechanism illustrated here may also be applicable to the process of Ca reabsorption by the kidney tubules (see text), with passive Ca entry into the tubule cells occurring across the brush border (lumenal) membrane, and Na–Ca exchange across the peritubular surface of the tubule cells (analogous to the serosal surface of the mucosal cells)

transepithelial potential (lumen positive to peritubular fluid—*cf.* BOULPAEP and SEELY, 1971). Moreover, since renal tubule cells are not impermeable to Ca (see below), and since the cytoplasm of proximal tubule cells is electrically negative to both lumenal and peritubular fluids (*cf.* BOULPAEP and SEELY, 1971; BOULPAEP, 1972), Ca may be expected to readily enter these cells across their lumenal borders. The energy-dependent step in Ca reabsorption must then occur at the peritubular surface of the tubule cells. The situation here is thus analogous to the Ca absorption process in the intestine (Section VI, A): and here, too, there is evidence that Na ions affect Ca transport.

Renal clearance (e.g. WALSER, 1961; KUPFER and KOSOVSKY, 1965; ANTONIOU et al., 1969; and see the review by WALSER, 1971), micropuncture (e.g. LASSITER et al., 1963; DUARTE and WATSON, 1967; MOREL et al., 1969; and see WALSER, 1971) and microperfusion studies (FRICK et al., 1965; BRUNETTE and ARAS, 1971) have consistently shown a striking correlation between the renal handling of Na and Ca, particularly in the proximal tubule, under a wide variety of salt loading and diuresis conditions. As Na excretion increases, so does the excretion of Ca-although the interrelationship between these two ions is probably not linear: KUPFER and KOSOVSKY (1965) found that cardiac glycosides greatly increased the excretion of both Na and Ca, and regraphing of their data indicates that the Ca clearance may be directly related to the square of the Na excretion (Fig. 16).

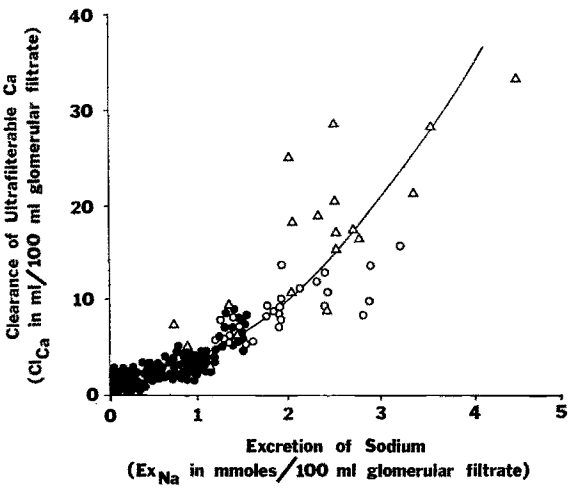


Fig. 16. Interrelationship between the clearance of ultrafilterable Ca (Cl_{Ca}, in ml per 100 ml of glomerular filtrate) and the excretion of sodium (Ex_{Na}, in mmoles per 100 ml of glomerular filtrate) in the dog kidney. The data of KUPFER and KOSOVSKY (1965) for three conditions, control (•), mannitol diuresis (○) and cardiac glycoside diuresis (△) have been combined and re-graphed; some of the control points (Na excretion values less than 1.0) have been omitted for the sake of clarity. The data have been fitted to the empirical equation:

$$Cl_{Ca} = 2 + (1.43\ Ex_{Na})^2$$

VOGEL and his colleagues have measured Ca reabsorption in the double-perfused, isolated frog kidney, in which the glomerulus and the peritubular space can be perfused independently, via the renal artery and renal portal vein, respectively. The Ca (and Na) reabsorption rate (from the glomerular filtrate) was dramatically decreased when both perfusion solutions contained the cardiac glycoside, convallatoxin, or the diuretic, furosemide (VOGEL and STOECKERT, 1967) or when K^+ was omitted from both solutions (VOGEL and TERVOOREN, 1965). When the Na in the renal portal vein was partially or completely replaced by mannitol, the rate of Ca re-absorption was decreased despite an increase in the urinary (tubular lumen) Ca concentration. However, the Ca reabsorption rate and Ca concentration in the urine were unaffected by complete replacement of Na in the renal artery perfusion solution—which enters the renal tubule as the glomerular filtrate (VOGEL and STOECKERT, 1967). Although not conclusive, these results indicate that the probable site of interaction between Ca and Na is the peritubular surface of the renal epithelium.

Experiments on kidney slices and isolated cortical tubule segments (*cf.* BURG and ORLOFF, 1963) add support to the idea that Na–Ca exchange may be involved in renal tubular transport of Ca. HOFER and KLEINZELLER (1963) noted that Ca uptake by rabbit renal cortex slices increased somewhat when the incubation medium Na was partially replaced by choline; Ca efflux from Ca-loaded slices was slightly reduced by a similar change in the composition of the medium. Fig. 17 shows that the rate of tracer efflux from ^{45}Ca-loaded renal cortical tubule

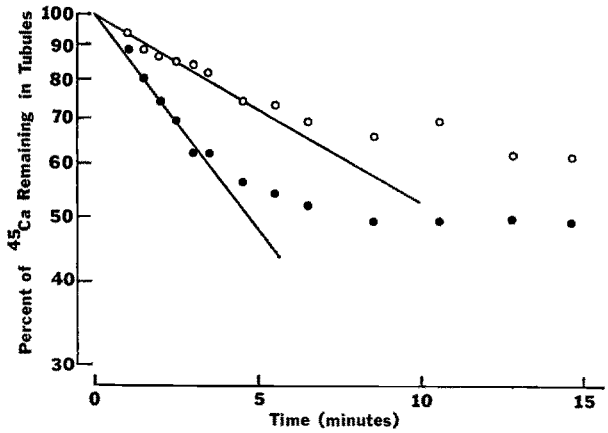


Fig. 17. Effect of external Na on the time-course of Ca efflux from ^{45}Ca-loaded isolated renal tubule segments. Rabbit renal cortical tubule segments, prepared by the method of BURG and ORLOFF (1962) were incubated in an ice-cold ^{45}Ca-containing solution to load them with isotope (*cf.* HOFER and KLEINZELLER, 1963). Extracellular isotope was removed by centrifuging the suspensions, decanting the supernatant solutions, and then washing the pellets twice (by resuspension and centrifugation) with ice-cold Ca-free, Na-free Li solution. The tubule segments were then resusupended either in Na-containing, or in Na-free Li-containing media (both Ca-free) and incubated at 30° C with continuous oxygenation. Aliquots of the susupension were sampled periodically, and the tubules and fluid were separated by filtration and assayed for radioactivity. As shown in the figure, the time-constant for ^{45}Ca efflux was increased from about 7 min to about 16 min when external Na was replaced by Li (BLAUSTEIN, unpublished data)

segments is reduced when external Na is replaced by Li; similar results are obtained when choline is substituted for Na (BLAUSTEIN, unpublished data). Conversely, Ca uptake by Na-loaded tubule segments is enhanced by Na_o-depletion (BLAUSTEIN, unpublished data).

The emerging picture of how Ca is reabsorbed by renal tubule cells is far from complete. Nevertheless, sufficient fragments are available to justify the speculation that perhaps the model used to explain Ca absorption in the intestine (Fig. 15 and see Section VI, A) may also be applicable to the kidney: Ca may diffuse into the renal tubule cells across the lumenal brush border, and be extruded across the peritubular surface by an Na–Ca exchange mechanism.

VII. Evidence of Na–Ca Exchange in Other Tissues

In concluding this survey of examples of possible Na–Ca exchange mechanisms, studies on several other tissues should also be mentioned. Of these, the most complete set of observations has been made on rat liver slices, which accumulate more Ca from Na-free (choline-substituted) media than from standard, Na-containing media (JUDAH and AHMED, 1963). The net efflux of Ca from liver slices is Na_o-dependent, and is inhibited by cardiac glycosides or K_o-depletion (JUDAH and AHMED, 1964; but see VAN ROSSUM, 1970).

The chorio-allantoic membrane of the embryonic chick is involved in active reabsorption of egg shell Ca for re-utilization by the embryo, and the net transport of Ca away from the ectodermal (shell) side of this membrane is inhibited by low concentrations of ouabain (TEREPKA et al., 1969). Higher concentrations of ouabain also inhibit Ca accumulation by this tissue (TEREPKA et al., 1969), as does deprivation of external Na (GARRISON and TEREPKA, 1972), but it is difficult to interpret these experiments because the site of Ca accumulation and the site (or side) of Na action have not yet been pinpointed in this directionally-oriented structure.

LISMAN and BROWN (1972) have examined the effects of intracellular Na and Ca on the response, to illumination, of the horseshoe crab (*Limulus*) ventral photoreceptor: The light response is diminished when either Ca or Na (but not K or Li) is injected iontophoretically, but the effect of Na injection is Ca_o-dependent. Prolonged treatment with strophanthidin or with K-free media also progressively decreases the light response, and these effects, too, are Ca_o-dependent (BROWN and LISMAN, 1972). All of these observations could be accounted for if the light response is a function of pCa, and if increasing $[Na]_i$ leads to a decrease in pCa by an Na–Ca exchange mechanism (LISMAN and BROWN, 1972).

Finally, CITTADINI et al. (1973) have noted that Ca accumulation by Ehrlich ascites tumor cells is enhanced when Na_o is replaced by sucrose and mannitol, but not by Li or K. Further investigation will be required to determine whether or not Ca fluxes in this preparation may involve Na–Ca exchange.

VIII. ATP-Dependent Calcium Fluxes and Na–Ca Exchange

As indicated above, several ATP-dependent Ca transport systems have been intensively studied in recent years. Although a detailed review of this subject is beyond the scope of the present article, the comparison of salient features of several types of Ca transport mechanisms given in Table 2 may help to place Na–Ca exchange in perspective, and to clarify some of the issues which were raised earlier-on. For the reader's convenience, the effects of various transport activators and inhibitors on the sodium pump (Na–K exchange) are also tabulated. It is apparent that each of these transport mechanisms is rather distinct in terms of its response to the agents listed in the first column of the table. In addition, two other properties of Na–Ca exchange may serve to further distinguish this mechanism from the others mentioned in the table: in contrast to all of the other transport systems, Na–Ca exchange does not appear to be inherently directionally-oriented. Rather, the direction of net Ca movement appears to depend largely upon the direction and magnitude of the Na concentration gradient. By way of contrast, the erythrocyte Ca pump and the sodium pumps of most cells are involved only in net extrusion of these cations, while the ATP-dependent Ca transport systems in mitochondria and sarcoplasmic reticulum are involved exclusively in the net accumulation of Ca by these organelles.

Table 2. Properties of calcium and sodium transport mechanisms

Activator or inhibitor	Na–Ca exchange	Mitochondrial Ca accumulation	Sarcoplasmic reticulum Ca accumulation	Red blood cell Ca extrusion	Sodium pump (Na–K and Na–Na exchange)
ATP	Not required, but may alter carrier affinity for Ca [1].	Required in the absence of oxidative metabolism [3].	Required [7]	Required [12]	Required for both Na–K and Na–Na exchange; hydrolyzed only in the case of Na–K exchange [14, 15, 16].
Na	Ca efflux proportional to $[Na]_o^3$, inhibited by Na_i; Ca influx proportional to $[Na]_i^3$ inhibited by Na_o [1].	No effect or slightly inhibitory; Ca efflux is stimulated by K, not Na, in the medium [3, 4, 5].	No effect [8].	No effect [12, 13].	Na efflux and K influx proportional to $[Na]_i$; Na competes with K_o [17, 18, 19, 20].
Oligomycin	No effect [1, 2].	Inhibits ATP-dependent Ca uptake in the absence of oxidative metabolism [3].	Inhibits Ca uptake [9].	No effect [12].	Inhibits Na extrusion and ATP hydrolysis [21].
Caffeine	No direct effect [1].	No effect [6].	Inhibits Ca uptake [6].	No effect [12].	—
Cardiac glycosides	No direct effect [1].	No effect [5].	No effect [5, 10].	No effect [12].	Inhibits Na–K and Na–Na exchange, and ATP hydrolysis [22].
Cyanide	No direct effect [1].	Releases stored Ca in the absence of exogenous ATP [3].	No direct effect [11].	—	No direct effect [14, 16].

References for Table 2.

1. Discussed in the present article. 2. Di Polo, 1973a. 3. Carafoli and Rossi, 1971. 4. Drahota and Lehninger, 1965. 5. Dransfeld et al., 1969. 6. Weber, 1968. 7. Hasselbach and Makinose, 1961. 8. Martinosi and Feretos, 1964. 9. Fairhurst et al., 1964. 10. Lee and Klaus, 1971. 11. Martonosi and Feretos, 1963. 12. Schatzmann and Vincenzi, 1969. 13. Blaustein, unpublished data. 14. Caldwell et al., 1960. 15. Garrahan and Glynn, 1967b. 16. Mullins and Brinley, 1967. 17. Garrahan and Glynn, 1967a. 18. Baker et al., 1969a. 19. Baker et al., 1969b. 20. Mullins and Brinley, 1969. 21. Blake et al., 1967. 22. Glynn, 1964.

Due to technical limitations, the voltage-dependence of transport has been examined only in the case of the sodium pump and Na–Ca exchange. Whereas Na–K exchange is insensitive to large changes in the transmembrane electric field (Brinley and Mullins, personal communication), Na–Ca exchange is voltage-sensitive (Section III, A, 1a). Although these observations may suggest that the energy available from ATP hydrolysis is sufficient to overcome a greater-than-

normal electrochemical gradient for Na, there is, of course, no indication as to whether or not the same situation might apply to the Ca electrochemical gradient in the case of ATP-dependent Ca transport mechanisms.

In sum, the foregoing considerations indicate that Na–Ca exchange is most likely an independent and mechanistically different process from the other transport mechanisms listed in Table 2.

IX. Possible Clinical Implications of Na–Ca Exchange

A. On the Positive Inotropic Action of Cardiac Glycosides

Cardiac glycosides have been used in the therapy of congestive heart failure for over two centuries. While their mechanism of action at the cellular level remains uncertain, SCHATZMANN (1953) made what is likely a giant step toward the solution of this problem with his observation that active transport of Na and K is inhibited by cardiac glycosides. Considerable evidence of the close correlation between inhibition of the sodium pump and the positive inotropic action of the cardiac glycosides has accumulated during the last two decades (*cf.* GLYNN, 1964; REITER, 1970; LEE and KLAUS, 1971). Inhibition of the sodium pump remains the only consistently-observed action of pharmacologic concentrations of cardiac glycosides (MÜLLER, 1965), although there have been occasional, disputed reports of an action of the glycosides on Ca transport by sarcoplasmic reticulum (compare DRANSFELD et al., 1969, and LEE, 1971).

If the inotropic action of the glycosides is, indeed, a consequence of (partial) inhibition of Na–K exchange, the question to be answered is: how are these two phenomena interrelated? BIRKS and COHEN (1965; 1968b) and BAKER et al. (1969a) have suggested that the Na–Ca exchange may provide the missing link, and REITER (1970) has shown that both Na_o and Ca_o play an important role in the inotropic effect. If Na–Ca exchange helps to maintain the Ca electrochemical gradient in normal, intact cardiac muscle (see section IV, B), partial inhibition of Na extrusion would result in a slight increase in $[Na]_i$, and *pari passu*, in $[Ca]_i$ (*cf.* Equation 3). The amount of Ca stored in the sarcoplasmic reticulum (and mitochondria) should also increase slightly, since accumulation of Ca by these organelles is dependent upon the ambient (sarcoplasmic) pCa (e.g. WEBER, 1966). The Ca-dependent increase in oxygen consumption of the resting (non-contracting) heart which results from exposure to low concentrations of cardiac glycosides (*e.g.* FINKELSTEIN and BODANSKY, 1948; and see LEE and KLAUS, 1971) might, in fact, be a manifestation of Ca-stimulated mitochondrial respiration (*cf.* LEHINGER et al., 1969; CARAFOLI and ROSSI, 1971) as a result of a decrease in sarcoplasmic pCa. The absolute magnitude of the Ca concentration changes may be quite small; no significant changes have been observed, although there is an increase in the "exchangeable" Ca (*cf.* LEE and KLAUS, 1971). Furthermore, there is general agreement that toxic concentrations of cardiac glycosides, which may simply

exaggerate the effects of lower concentrations, do significantly increase cell Ca levels (*cf.* LEE and KLAUS, 1971).

Since the relation between tension and pCa is sigmoid, with no tension developed at high pCa, a small decrease in pCa should have a negligible effect on tension in relaxed (resting) muscle. However, in the range of twitch tensions, where tension is inversely proportional to pCa (*cf.* WINEGRAD, 1971), a small increment in the amount of Ca released from sarcoplasmic reticulum (assuming that the amount released is proportional to the amount stored) during excitation might significantly increase contractile tension.

The possibility that Na–Ca exchange may link the inotropic action of cardiac glycosides to their inhibition of the sodium pump is thus an attractive hypothesis which fits most of the available data. However, this hypothesis obviously rests on several tenuous assumptions, and many gaps in our knowledge remain to be filled in.

B. Does Na–Ca Exchange Play a Role in Some Forms of Hypertension?

In experimental renal and endocrine hypertension, the Na content of the vascular wall is elevated (SCHOFFENIELS, 1969), but it is not known whether at least a part of this increase represents an increase in the $[Na]_i$ of the smooth muscle fibers. If the $[Na]_i$ does increase, one can speculate that pCa may then decline as a result of Na–Ca exchange (*cf.* REUTER et al., 1973). Evidence that Na–Ca exchange may be involved in the regulation of resting pCa, and consequently, steady tension in arterial smooth muscle, was discussed in Section IV, C. Assuming that slight alterations in $[Na]_i$ may indeed affect pCa and tension in this tissue, Na–Ca exchange may thus play a critical role in the development of some forms of hypertension. Incidentally, these considerations may also indicate why restricted Na intake and the administration of saluretics are frequently effective therapy for mild hypertension.

X. Summary and Conclusions

A. What is the Source of Energy for Calcium Extrusion?

Available evidence indicates that most animal cells must extrude Ca against a large electrochemical gradient in order to remain in steady Ca balance. In many types of cells a portion of the transmembrane Ca fluxes appears to be coupled to a counterflow of Na^+ ions. Under these circumstances, the Na gradient could supply some of the energy required to actively extrude Ca. The most detailed kinetic data are available for nerve and muscle, and in some preparations (squid axon, barnacle muscle, synaptosomes) these data indicate that the stoichiometry of the exchange may be: three Na^+ entering-for-one Ca^{2+} leaving. Ca efflux from the squid axon (data are not available for other preparations) also is driven, in part, by the

electrical potential gradient. The total contribution of the Na and potential gradients could in theory, supply sufficient energy to maintain the observed Ca gradient. But, as already pointed out, these considerations do not necessarily rule out direct participation of ATP or other high-energy metabolic intermediates in Ca extrusion via Na–Ca exchange. The fact that metabolic poisons such as cyanide and 2,4-dinitrophenol stimulate Na_o-dependent Ca efflux in some preparations may be additional evidence that ATP is not required. However, it must be remembered that these poisons release Ca from intracellular stores, thereby providing a more favorable transmembrane gradient for Ca extrusion. We are therefore forced to conclude that the question of whether or not high-energy metabolic intermediates participate directly in Na–Ca exchange is, as yet, unresolved.

B. Does Na–Ca Exchange Help to Regulate pCa in Intact Cells?

Although it seems clear that a variety of cell types apparently do possess Na–Ca exchange mechanisms, it is important to know whether or not these mechanisms participate in the regulation of pCa. In most of the experiments described in the preceding sections, normal physiologic conditions were significantly altered in order to obtain evidence of Na–Ca exchange. In a few instances, however, conditions which may only slightly alter the distribution of Na also appear to affect pCa (*e.g.* see Section IX). Thus, the evidence that Na–Ca exchange is involved in the regulation of pCa is suggestive but not conclusive.

A possibly related problem stems from the fact that perhaps as much as 25–40 per cent of the total Ca efflux from squid axon and barnacle muscle (and presumably other tissues, from other animals, as well) is apparently Ca_o- and Na_o-independent. This flux is too large to be accounted for by passive diffusion. One hypothetical explanation is that the "residual" Ca efflux may be the manifestation of a separate, energy-dependent Ca extrusion mechanism which operates in parallel with the Na–Ca exchange carrier. But, aside from the lack of experimental evidence, such a duplication of effort seems rather unlikely, unless insufficient energy (in the form of Na gradients, potential gradients, and perhaps ATP) is available to the Na–Ca exchange carriers to maintain the physiological pCa. These considerations leave the enigma of the Na_o- and Ca_o-independent Ca efflux unsettled. A possible clue that it may not however, be associated with a separate transport mechanism comes from the observation (RUSSELL and BLAUSTEIN, 1974) that low concentrations of caffeine (insufficient to induce contractures) in barnacle muscle increase the Na_o-dependent Ca efflux at the expense of the "residual" Ca efflux – presumably as a consequence of a slight fall in pCa.

On the basis of available evidence, it seems reasonable to conclude that the widely-distributed Na–Ca exchange mechanisms probably do play a role in pCa regulation in a variety of cell types. But we must recognize that the story has not yet unfolded completely. The directions which future research must take in order to rigorously test this hypothesis are obvious, and if this view survives the test of time, it may help to tie together many of the seemingly-unrelated observations described above.

XI. Appendix: Calculation of the Calcium Ion Activity Coefficient

The activity coefficient of any ion in dilute solution depends soley upon the total ionic strength of the solution (LEWIS and RANDALL, 1921). It should therefore be possible to calculate approximate activity coefficients for individual ion species in mixed electrolyte solutions such as physiological salt solutions. To a first approximation, it seems reasonable to assume that extracellular fluids such as human blood plasma and squid hemolymph are primarily mixtures of NaCl, $MgCl_2$, and $CaCl_2$ provided that appropriate corrections are made for the contributions of other ions to the total ionic strength (I; Equation 4c) of the solution. Activity coefficients may then be calculated from the Debye-Hückel-Güntelberg equation, as modified by GUGGENHEIM (ROBINSON and STOKES, 1968, page 231):

$$\log f_{\pm} = -\frac{A|z_+ z_-|\sqrt{I}}{1+\sqrt{I}} + b(I) \tag{4}$$

where the mean activity coefficient, $f_{\pm}$ is given by:

$$f_+^{\nu} = f_+^{\nu_+} f_-^{\nu_-} \tag{4a}$$

and

$$\nu = \nu_+ + \nu_- \tag{4b}$$

where ν_+ and ν_- are the numbers of moles of cation and anion, respectively, formed from one mole of electrolyte.

In Equation (4), I denotes the total ionic stength of the solution:

$$I = {}^1/_2 \sum c_i z_i^2 \tag{4c}$$

where c_i is the concentration (in moles per liter) of the i^{th} ion, whose valence is z. The z_+ and z_- in equation 4 refer to the valence of the cation and anion, respectively. The constant, A, in Equation (4) involves the absolute temperature and the dielectric constant of the solvent; for aqueous solutions it has a value of 0.50 at 15° C and 0.52 at 35° C (ROBINSON and STOKES, 1968, page 468). The second constant, b, may be approximated by $0.1|z_+ z_-|$ (DAVIES, 1938, quoted by ROBINSON and STOKES, 1968).

The mean activity coefficients for NaCl and for $CaCl_2$ can be calculated by inserting the appropriate values (see Table 1) into Equations (4) and (4a) through (4c). Values for the Ca activity coefficient of 0.30 and 0.23 for human blood plasma and squid hemolymph, respectively, are then obtainable from Equation (4a), if it is assumed that the activity coefficients for Na and Cl are equal[4]. As indicated in Section II A, these values are in reasonably good agreement with the single ion activity coefficients calculated from hydration theory (BATES et al., 1970).

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[4] Calculations of individual ion activity coefficients from hydration theory indicate that this approximation should err by less than 10 per cent in the range of NaCl concentrations employed here.

References

ADAMS, T. H., NORMAN, A. W.: Studies on the mechanism of action of calciferol. I. Basic parameters of vitamin D-mediated calcium transport. J. biol. Chem. **245**, 4421–4431 (1970).

ANTONIOU, L. D., EISNER, G. M., SLOTKOFF, L. M., LILIENFIELD, L. S.: Relationship between sodium and calcium transport in the kidney. J. Lab. clin. Med. **74**, 410–420 (1969).

ARMSTRONG, C. M., BEZANILLA, F. M., HOROWICZ, P.: Twitches in the presence of ethylene glycol bis (β-aminoethyl ether)-N,N'-tetraacetic acid. Biochim. biophys. Acta (Amst.) **267**, 605–608 (1972).

ASHLEY, C. C., CALDWELL, P. C., LOWE, A. G.: The efflux of calcium from single crab and barnacle muscle fibres. J. Physiol. (Lond.) **223**, 737–755 (1972).

BAKER, P. F.: Transport and metabolism of calcium ions in nerve. Prog. Biophys. molec. Biol. **24**, 177–223 (1972).

BAKER, P. F., BLAUSTEIN, M. P.: Sodium-dependent uptake of calcium by crab nerve. Biochim. biophys. Acta (Amst.) **150**, 167–170 (1968).

BAKER, P. F., BLAUSTEIN, M. P., HODGKIN, A. L., STEINHARDT, R. A.: The influence of calcium on sodium efflux in squid axons. J. Physiol. (Lond.) **200**, 431–458 (1969a).

BAKER, P. F., BLAUSTEIN, M. P., KEYNES, R. D., MANIL, J., SHAW, T. I., STEINHARDT, R. D.: The ouabain-sensitive fluxes of sodium and potassium in squid giant axons. J. Physiol. (Lond.) **200**, 459–496 (1969b).

BAKER, P. F., CRAWFORD, A. C.: Mobility and transport of magnesium in squid giant axons. J. Physiol. (Lond.) **227**, 855–874 (1972).

BAKER, P. F., GLITSCH, H. G.: Does metabolic energy participate directly in the Na^+-dependent extrusion of Ca^{2+} ions from squid giant axons? J. Physiol. (Lond.) **233**, 44–46 p (1973).

BAKER, P. F., HODGKIN, A. L., RIDGEWAY, E. B.: Depolarization and calcium entry in squid axons. J. Physiol. (Lond.) **218**, 709–755 (1971).

BANKS, P.: The effect of ouabain on the secretion of catecholamines and on the intracellular concentration of potassium. J. Physiol. (Lond.) **193**, 631–637 (1967).

BANKS, P., BIGGINS, R., BISHOP, R., CHRISTIAN, B., CURRIE, N.: Sodium ions and the secretion of catecholamines. J. Physiol. (Lond.) **200**, 797–805 (1969).

BATES, R. G., STAPLES, B. R., ROBINSON, R. A.: Ionic hydration and single ion activities in unassociated chlorides at high ionic strengths. Anal. Chem. **42**, 867–871 (1970).

BIAMINO, G., JOHANSSON, B.: Effects of calcium and sodium on contracture tension in the smooth muscle of the rat portal vein. Pflügers Arch. ges. Physiol. **321**, 143–158 (1970).

BIANCHI, C. P.: Cell Calcium. London: Butterworths 1968.

BIANCHI, C. P., SHANES, A. M.: Calcium influx in skeletal muscle at rest, during activity, and during potassium contracture. J. gen. Physiol. **42**, 805–815 (1959).

BIRKS, R. I., COHEN, M. W.: Effects of sodium on transmitter release from frog motor nerve terminals. In: PAUL, W. M., DANIEL, E. E., KAY, C. M., MONKTON, G.: Muscle, p. 403–420. London Pergamon Press 1965.

BIRKS, R. I., COHEN, M. W.: The action of sodium pump inhibitors on neuromuscular transmission. Proc. roy Soc. B **170**, 381–399 (1968a).

BIRKS, R. I., COHEN, M. W.: The influence of internal sodium on the behaviour of motor nerve terminals. Proc. roy Soc. B **170**, 401–421 (1968b).

BLAKE, A., LEADER, D. P., WHITTAM, R.: Physical and chemical reactions of phosphates in red cell membranes in relation to active transport. J. Physiol. (Lond.) **193**, 467–479 (1967).

BLASZKOWSKI, T. P., BOGDANSKI, D. F.: Possible role of sodium and calcium ions in retention and physiological release of norepinephrine by adrenergic nerve endings. Biochem. Pharmacol. **20**, 3281–3294 (1971).

BLAUSTEIN, M. P.: Preganglionic stimulation increases calcium uptake by sympathetic ganglia. Science **172**, 391–393 (1971).

BLAUSTEIN, M. P., HODGKIN, A. L.: The effect of cyanide on the efflux of calcium from squid axons. J. Physiol. (Lond.) **200**, 497–527 (1969).

BLAUSTEIN, M. P., JOHNSON, E. M., JR., NEEDLEMAN, P.: Calcium-dependent norepinephrine release from presynaptic nerve endings *in vitro*. Proc. nat. Acad. Sci. (Wash.) **69**, 2237–2240 (1972).

BLAUSTEIN, M. P., OBORN, C. J.: The influence of sodium on calcium fluxes in pinched-off nerve terminals *in vitro*. Manuscript in preparation.

BLAUSTEIN, M. P., RUSSELL, J. M.: Calcium efflux from barnacle muscle fibers: Dependence on external cations. Manuscript in preparation.

BLAUSTEIN, M. P., RUSSELL, J. M., DEWEER, P.: Calcium efflux from internally-dialyzed squid axons: the influence of external and internal cations. J. Supramolec. Struct., in press.

BLAUSTEIN, M. P., WIESMANN, W. P.: Effect of sodium ions on calcium movements in isolated synaptic terminals. Proc. nat. Acad. Sci. (Wash.) **66**, 664–671 (1970).

BOHR, D. F.: Electrolytes and smooth muscle contraction. Pharmacol. Rev. **16**, 85–111 (1964).

BORLE, A. B.: Membrane transfer of calcium. Clin. Orthop. **52**, 267–291 (1967).

BORLE, A. B.: Kinetic analysis of calcium movements in HeLa cell cultures. II. Calcium efflux. J. gen. Physiol. **53**, 57–69 (1969).

BOULPAEP, E. L.: Permeability changes of the proximal tubule of *Necturus* during saline loading. Amer. J. Physiol. **222**, 517–531 (1972).

BOULPAEP, E. L., SEELY, J. F.: Electrophysiology of proximal and distal tubules in the autoperfused dog kidney. Amer. J. Physiol. **221**, 1084–1096 (1971).

BRINK, F.: The role of calcium ions in neural processes. Pharmacol. Rev. **6**, 243–298 (1954).

BRINLEY, F. J., JR.: Sodium and potassium fluxes in isolated barnacle muscle fibers. J. gen. Physiol. **51**, 445–477 (1968).

BRINLEY, F. J., JR., MULLINS, L. J.: Sodium extrusion by internally dialyzed squid axons. J. gen. Physiol. **50**, 2303–2331 (1967).

BROWN, J. E., LISMAN, J. E.: An electrogenic sodium pump in *Limulus* ventral photoreceptor cells. J. gen. Physiol. **59**, 720–733 (1970).

BRUNETTE, M., ARAS, M.: A microinjection study of nephron permeability to calcium and magnesium. Amer. J. Physiol. **221**, 1442–1448 (1971).

BULL, R. J., TREVOR, A. J.: Sodium and the flux of calcium ions in electrically-stimulated cerebral tissue. J. Neurochem. **19**, 1011–1022 (1972).

BURG, M. B., ORLOFF, J.: Oxygen consumption and active transport in separated renal tubules. Amer. J. Physiol. **203**, 327–330 (1963).

CALDWELL, P. C.: The phosphorus metabolism of squid axons and its relationship to the active transport of sodium. J. Physiol. (Lond.) **152**, 545–560 (1960).

CALDWELL, P. C.: Energy relationships and the active transport of ions. Curr. Top. Bioenerget. **3**, 251–278 (1969).

CALDWELL, P. C., KEYNES, R. D.: The effect of ouabain on the efflux of sodium from a squid giant axon. J. Physiol. (Lond.) **148**, 8–9P (1959).

CALDWELL, P. C., HODGKIN, A. L., KEYNES, R. D., SHAW, T. I.: The effects of injecting energy-rich phosphate compounds on the active transport of ions in the giant axon of *Loligo*. J. Physiol. (Lond.) **152**, 561–590 (1960).

CARAFOLI, E.: *In vivo* effect of uncoupling agents on the incorporation of calcium and strontium into mitochondria and other subcellular fractions of rat liver. J. gen. Physiol. **50**, 1849–1864 (1967).

CARAFOLI, E., ROSSI, C. S.: Calcium transport in mitochondria. Adv. Cytopharmacol. **1**, 209–227 (1971).

CASSIDY, M. M., GOLDNER, A. M., TIDBALL, C. S.: Subcellular localization of calcium, magnesium and protein in canine intestinal mucosa. Amer. J. Physiol. **217**, 680–685 (1969).

CASTEELS, R., RAEYMAEKERS, L., GOFFIN, J., WUYTAK, F.: A study of factors affecting the cellular calcium content of smooth muscle cells. Arch. int. Pharmacodyn. **201**, 191–192 (1973).

CITTADINI, A., SCARPA, A., CHANCE, B.: Calcium transport in intact Ehrlich ascites tumor cells. Biochim. biophys. Acta (Amst.) **291**, 246–259 (1973).

COMAR, C. L., BRONNER, F. (Eds.): Mineral Metabolism, Volume III: Calcium Physiology. New York: Academic Press 1971.

COOKE, W. J., ROBINSON, J. D.: Factors influencing calcium movements in rat brain slices. Amer. J. Physiol. **221**, 218–225 (1971).

COPE, F. W.: Spin-echo nuclear magnetic resonance evidence for complexing of sodium ions in muscle, brain and kidney. Biophys. J. **10**, 843–858 (1970).

COSMOS, E. E., HARRIS, E. J.: *In vitro* studies of the gain and exchange of calcium in frog skeletal muscle. J. gen. Physiol. **44**, 1121–1130 (1961).

COSTANTIN, L. L.: Activation in striated muscle. In KANDEL, E. (Ed.): Handbook of Physiology. Washington: American Physiological Society 1974, in press.

CSAKY, T. Z., HARA, Y.: Inhibition of active intestinal sugar transport by digitalis. Amer. J. Physiol. **209**, 467–472 (1965).

CURTIS, B. A.: Ca fluxes in single twitch muscle fibers. J. gen. Physiol. **50**, 255–267 (1966).

DEAN, P. M., MATTHEWS, E. K.: Electrical activity in pancreatic islet cells. Nature (Lond.) **219**, 389–390 (1968).

DEVINE, C. E., SOMLYO, A. V., SOMLYO, A. P.: Sarcoplasmic reticulum and excitation-contraction coupling in mammalian smooth muscles. J. Cell Biol. **52**, 690–718 (1972).

DE WEER, P. J.: Aspects of the recovery processes in nerve. Int. Rev. Physiol. Sec. on Neurophysiology. In press 1974.

DICKER, S. E.: Release of vasopressin and oxytocin from isolated pituitary glands of adult and new-born rats. J. Physiol. (Lond.) **185**, 429–444 (1966).

DI POLO, R.: Calcium efflux from internally dialized squid giant axons. J. gen. Physiol. **62**, 575–589 (1973a).

DI POLO, R.: Sodium-dependent calcium influx in dialyzed barnacle muscle fibers. Biochim. biophys. Acta (Amst.) **298**, 279–283 (1973b).

DITTMER, D. S. (Ed.): Blood and other body fluids. Washington: Federation of American Societies for Experimental Biology 1961.

DONNAN, F. G.: The theory of membrane equilibria. Chem. Rev. **1**, 73–90 (1925).

DOUGLAS, W. W.: Stimulus-secretion coupling: the concept and clues from chromaffin and other cells. Brit. J. Pharmacol. **34**, 451–474 (1968).

DOUGLAS, W. W., KANNO, T., SAMPSON, S. R.: Effects of acetylcholine and other medullary secretagogues and antagonists on the membrane potential of adrenal chromaffin cells: An analysis employing techniques of tissue culture. J. Physiol. (Lond.) **188**, 107–120 (1967a).

DOUGLAS, W. W., KANNO, T., SAMPSON, S. R.: Influence of the ionic environment on the membrane potential of adrenal chromaffin cells and on the depolarizing effect of acetylcholine. J. Physiol. (Lond.) **191**, 107–121 (1967b).

DOUGLAS, W. W., RUBIN, R. P.: The role of calcium in the secretory response of the adrenal medulla to acetylcholine. J. Physiol. (Lond.) **159**, 40–57 (1961).

DOUGLAS, W. W., RUBIN, R. P.: The mechanism of catecholamine release from the adrenal medulla and the role of calcium in stimulus-secretion coupling. J. Physiol. (Lond.) **167**, 288–310 (1963).

DRAHOTA, Z., LEHNINGER, A. L.: Movements of H^+, K^+ and Na^+ during energy-dependent uptake and retention of Ca^{++} in rat liver mitochondria. Biochem. biophys. Res. Commun. **19**, 351–356 (1965).

DRAHOTA, Z., CARAFOLI, E., ROSSI, C. S., GAMBLE, R. L., LEHINGER, A. L.: The steady-state maintenance of accumulated Ca^{++} in rat liver mitochondria. J. biol. Chem. **240**, 2712–2720 (1965).

DRANSFELD, H., GREEF, K., SCHORN, A., TING, B. T.: Calcium uptake in mitochondria and vesicles of heart and skeletal muscle in presence of potassium, sodium, k-strophanthin and pentobarbital. Biochem. Pharmacol. **18**, 1335–1345 (1969).

EBASHI, S.: Calcium binding activity of vesicular relaxing factor. J. Biochem. (Tokyo) **50**, 236–244 (1961).

EBASHI, S., ENDO, M.: Calcium ion and muscle contraction. Progr. Biophys. molec. Biol. **18**, 123–183 (1968).

EBASHI, S., ENDO, M., OHTSUKI, I.: Control of muscle contraction. Quart. Rev. Biophys. **2**, 351–384 (1969).

EDWARDS, C., HARRIS, E. J.: Factors influencing the sodium movement in frog muscle with a discussion of the mechanism of sodium movement. J. Physiol. (Lond.) **135**, 567–580 (1957).

FAIRHURST, A. S., ELISON, C., JENDEN, D. J.: Inhibition of calcium transport in muscle granules by oligomycin. Life Sci. **3**, 953–958 (1964).

FILO, R. S., BOHR, D. F., RUEGG, J. C.: Glycerinated skeletal and smooth muscle: calcium and magnesium dependence. Science **147**, 1581–1583 (1965).

FINKELSTEIN, M., BODANSKY, O.: The effect of cardiac glycosides on the respiration of cardiac muscle. J. Pharmacol. exp. Ther. **94**, 274–287 (1948).

FITZPATRICK, D. F., LANDON, E. J., DEBBAS, G., HURWITZ, L.: A calcium pump in vascular smooth muscle. Science **176**, 305–306 (1972).

FLEISCHER, N., WOOD, J. MCM.: Ouabain stimulation of $^{45}Ca^{++}$ accumulation in the rat pituitary. Endocrinology **92**, 1555–1559 (1973).

FLEISCHER, N., ZIMMERMAN, G., SCHINDLER, W., HUTCHINS, M.: Stimulation of adrenocorticotropin (ACTH) and growth hormone (GH) release by ouabain: Relationship to calcium. Endocrinology **91**, 1436–1441.

FRANKENHAEUSER, B., HODGKIN, A. L.: The action of calcium on the electrical properties of squid axons. J. Physiol. (Lond.) **137**, 218–244 (1957).

FRICK, A., RUMRICH, G., ULLRICH, K. J., LASSITER, W. E.: Microperfusion study of calcium transport in the proximal tubule of the rat kidney. Pflügers Arch. ges. Physiol. **286**, 109–117 (1965).

FRIEDMAN, S. M., WONG, S. L., WALTON, J. H.: Gass electrode measurements of blood sodium and potassium in man. J. appl. Physiol. **18**, 950–954 (1963).

FRÖMTER, E., DIAMOND, J. M.: Route of passive ion permeation in epithelia. Nature New Biology (Lond.) **235**, 9–13 (1972).

FUJISAWA, H., KAJIKAWA, K., OHI, Y., HASHIMOTO, Y., YOSHIDA, H.: Movement of radioactive calcium in brain slices and influences on it of protoveratrine, ouabain, potassium chloride and cocaine. J. Pharmacol. (Kyoto) **15**, 327–334 (1965).

GAGE, P. W., QUASTEL, D. M. J.: Competition between sodium and calcium ions in transmitter release at mammalian nueromuscular junctions. J. Physiol. (Lond.) **185**, 95–123 (1966).

GARCIA, A. G., KIRPEKAR, S. M.: Release of noradrenaline from the cat spleen by sodium deprivation. Brit. J. Pharmacol. **47**, 729–747 (1973).

GARDOS, C.: The role of calcium in the permeability of human erythrocytes. Acta physiol. Acad. Sci. hung. **15**, 121–125 (1959).

GARRAHAN, P. J., GLYNN, I. M.: The sensitivity of the sodium pump to external sodium. J. Physiol. (Lond.) **192**, 175–188 (1967a).

GARRAHAN, P. J., GLYNN, I. M.: Factors affecting the relative magnitudes of the sodium: potassium and sodium: sodium exchanges catalysed by the sodium pump. J. Physiol. (Lond.) **192**, 189–216 (1967b).

GARRISON, J. C., TEREPKA, A. R.: The interrelationships between sodium ion, calcium transport and oxygen utilization in the isolated chick chorioallantoic membrane. J. Membr. Biol. **7**, 146–163 (1972).

GAYTON, D. C., ALLEN, R. D., HINKE, J. A. M.: The intracellular concentration and activity of sodium in giant barnacle muscle fibers. J. gen. Physiol. **54**, 433–435 (1969).

GECK, P., HEINZ, E., PFEIFFER, B.: The degree and efficiency of coupling between the influxes of Na^+ and α-aminoisobutyrate in Ehrlich cells. Biochim. biophys. Acta (Amst.) **288**, 486–491 (1972).

GIBB, L. E., EDDY, A. A.: An electrogenic sodium pump as a possible factor leading to the concentration of amino acids by mouse ascites-tumour cells with reversed sodium ion concentration gradients. Biochem. J. **129**, 979–981 (1972).

GIBBONS, R. A., SANSOM, B. F., SELLWOOD, R.: The passage of calcium and strontium across the gut of the anesthetized goat. J. Physiol. (Lond.) **222**, 397–406 (1972).

GLITSCH, H. G., REUTER, H., SCHOLZ, H.: The effect of the internal sodium concentration on calcium fluxes in isolated guinea-pig auricles. J. Physiol. (Lond.) **209**, 25–43 (1970).

GLYNN, I. M.: The action of cardiac glycosides on ion movements. Pharmacol. Rev. **16**, 381–407 (1964).

GOLDRING, J. M., BLAUSTEIN, M. P.: Synaptosome membrane potential changes monitored with a fluorescent probe. Society for Neuroscience sample Expanded Abstracts. Los Angeles: Brain Information service, p. 15 (1973).

GOODFORD, P. J.: The calcium content of the smooth muscle of the guinea-pig taenia coli. J. Physiol. (Lond.) **192**, 145–157 (1967).

HAGIWARA, S., NAKAJIMA, S.: Effects of the intracellular Ca ion concentration upon excitability of the muscle fiber membrane of a barnacle. J. gen. Physiol. **49**, 807–818 (1966).

HALES, C. N., MILNER, R. D. G.: The role of sodium and potassium in insulin secretion from rabbit pancreas. J. Physiol. (Lond.) **194**, 725–743 (1968a).

HALES, C. N., MILNER, R. D. G.: Cations and the secretion of insulin from rabbit pancreas in vitro. J. Physiol. (Lond.) **199**, 177–187 (1968b).

HANIG, R. C., TACHIKI, K. H., APRISON, M. H.: Subcellular distribution of potassium, sodium, magnesium, calcium and chloride in cerebral cortex. J. Neurochem. **19**, 1501–1507.

HASSELBACH, W., MAKINOSE, M.: Die Calciumpumpe der „Erschlaffungsgrana" des Muskels und ihre Abhängigkeit von der ATP-Spaltung. Biochem. Z. **333**, 518–528 (1961).

HAYES, F. R., PELLUET, D.: The inorganic constitution of molluscan blood and muscle. J. Marine Biol. Assoc. U.K. **26**, 580–589 (1947).

HELLAM, D. C., PODOLSKY, R. J.: Force measurements in skinned muscle fibres. J. Physiol. (Lond.) **200**, 807–819 (1969).

HIGGINS, C. B., VATNER, S. F., BRAUNWALD, E.: Regional hemodynamic effects of a digitalis glycoside in the conscious dog with and without experimental heart failure. Circulat. Res. **30**, 406–417 (1973).

HINKE, J. A. M.: The measurement of sodium and potassium activities in the squid axon by means of cation-selective glass micro electrodes. J. Physiol. (Lond.) **156**, 314–335 (1961).

HINKE, J. A. M., WILSON, M. L.: Effects of electrolytes on contractility of artery segments in vitro. Amer. J. Physiol. **203**, 1161–1166 (1962).

HODGKIN, A. L., KEYNES, R. D.: Active transport of cations in giant axons from *Sepia* and *Loligo*. J. Physiol. (Lond.) **128**, 28–60 (1955).

HODGKIN, A. L., KEYNES, R. D.: Movements of labelled calcium in squid axons. J. Physiol. (Lond.) **138**, 253–281 (1957).

HÖFER, M., KLEINZELLER, A.: Calcium transport in slices of rabbit kidney cortex: The loss of calcium from Ca-enriched slices. Physiol. bohemoslov. **12**, 425–434 (1963).

JANSEN, J. K. S., NICHOLLS, J. G.: Conductance changes, an electrogenic pump and the hyperpolarization of leech neurons following impulses. J. Physiol. (Lond.) **229**, 635–655 (1973).

JUDAH, J. D., AHMED, K.: Role of phosphoproteins in ion transport: Interactions of sodium with calcium and potassium in liver slices. Biochim. biophys. Acta (Amst.) **71**, 34–44 (1963).

JUDAH, J. D., AHMED, K.: The biochemistry of sodium transport. Biol. Rev. **39**, 160–193 (1964).

JUDAH, J. D., WILLOUGHBY, D. A.: Inhibitors of sodium dependent relaxation of guinea-pig ileum. J. cell. comp. Physiol. **64**, 363–370 (1964).

JULIAN, F. J.: The effect of calcium on the force-velocity relation of briefly glycerinated frog muscle fibres. J. Physiol. (Lond.) **218**, 117–145 (1971).

KALIX, P.: Uptake and release of calcium in rabbit vagus nerve. Pflügers Arch. ges. Physiol. **326**, 1–14 (1971).

KATASE, T., TOMITA, T.: Influences of sodium and calcium on the recovery process from potassium contracture in the guinea-pig taenia coli. J. Physiol. (Lond.) **224**, 489–500 (1972).

KATZ, B.: The Release of Neural Transmitter Substances. Liverpool University Press 1969.

KATZ, B., MILEDI, R.: A study of synaptic transmission in the absence of nerve impulses. J. Phy siol. (Lond.) **192**, 407–436 (1967).

KATZ, B., MILEDI, R.: Tetrodotoxin-resistant electric activity in presynaptic terminals. J. Physiol. (Lond.) **203**, 459–487 (1969).

KATZ, B., MILEDI, R.: Further study of the role of calcium in synaptic transmission. J. Physiol. (Lond.) **207**, 789–801 (1970).

KELLY, J. S.: Antagonism between Na^+ and Ca^+ at the neuromuscular junction. Nature (Lond.) **205**, 296–297.

KEYNES, R. D., LEWIS, P. R.: The intracellular calcium contents of some invertebrate nerves. J. Physiol. (Lond.) **134**, 399–407 (1956).

KEYNES, R. D., RITCHIE, J. M.: The movements of labelled ions in mammalian non-myelinated nerve fibres. J. Physiol. (Lond.) **179**, 333–367 (1965).

KIRPEKAR, S. M., WAKADE, A. R.: Release of noradrenaline from the cat spleen by potassium. J. Physiol. (Lond.) **194**, 595–608 (1968).

KOECHLIN, B. A.: On the chemical composition of the axoplasm of squid giant nerve fibers with particular reference to its ion pattern. J. biophys. biochem. Cytol. **1**, 511–529 (1955).

KREGENOW, F. M., HOFFMAN, J. F.: Some kinetic and metabolic characteristics of calcium-induced potassium transport in human red cells. J. gen. Physiol. **60**, 406–429 (1972).

KRNJEVIC, K., LISIEWICZ, A.: Injection of calcium ions into spinal motoneurones. J. Physiol. (Lond.) **225**, 363–390 (1972).

KUPFER, S., KOSOVSKY, J. D.: Effects of cardiac glycosides on renal tubular transport of calcium, magnesium, inorganic phosphate and glucose in the dog. J. clin. Invest. **44**, 1132–1143 (1965).

KURIYAMA, H.: Effects of ions and drugs on the electrical activity of smooth muscle. In: BULBRING, E., A. F. BRADING, A. W. JONES, T. TOMITA (Eds.): Smooth Muscle, p. 366–395. Baltimore: Williams and Wilkins 1970.

LAMBERT, A. E., JEANRENAUD, B., JUNOD, A., RENOLD, A. E.: Organ culture of fetal rat pancreas. II. Insulin release induced by amino and organic acids, by hormonal peptides, by cationic alterations in the medium and by other agents. Biochim. biophys. Acta (Amst.) **174**, 540–553 (1969).

LASSITER, W. E., GOTTSCHALK, C. W., MYLLE, M.: Micropuncture study of renal tubular reabsorption of calcium in normal rodents. Amer. J. Physiol. **204**, 771–775 (1963).

LAUTERBACH, F.: Beziehungen zwischen enteraler Resorption aktiv transportierter sowie diffundierender Substanzen und Konzentration sowie Transport von Na^+-Ionen. Biochim. biophys. Acta (Amst.) **135**, 256–272 (1967a).

LAUTERBACH, F.: Die Wirkung cardiotoner Steroide auf die enterale Resorption aktiv transportierter und diffundierender Substanzen und auf deren Beziehung zu Na^+-Konzentration und Na^+-Transport. Biochim. biophys. Acta (Amst.) **135**, 273–285 (1967b).

LEE, K. S., KLAUS, W.: The subcellular basis for the mechanism of inotropic action of cardiac glycosides. Pharmacol. Rev. **23**, 193–261 (1971).

LEHNINGER, A. L.: Mitochondria and calcium ion transport. Biochem. J. **119**, 129–138 (1970).

LEHNINGER, A. L., CARAFOLI, E., ROSSI, C. S.: Energy-linked ion movements in mitochondrial systems. Adv. Enzymol. **29**, 259–320 (1967).

LEONARD, E.: Alteration of contractile response of artery strips by a potassium-free solution, cardiac glycosides and changes in stimulus frequency. Amer. J. Physiol. (Lond.) **189**, 185–190 (1957).

LEWIS, G. N., RANDALL, M.: The activity coefficient of strong electrolytes. J. Amer. chem. Soc. **43**, 1112–1154 (1921).

LISMAN, J. E., BROWN, J. E.: The effects of intracellular iontophoretic injection of calcium and sodium ions on the light response of *Limulus* ventral photoreceptors. J. gen. Physiol. **59**, 701–719 (1972).

LLINÁS, R., BLINKS, J. R., NICHOLSON, C.: Calcium transient in presynaptic terminal of squid giant synapse: Detection with aequorin. Science **176**, 1127–1129 (1972).

LOEWENSTEIN, W.: Membrane junctions in growth and differentiation. Fed. Proc. **32**, 60–64 (1973).

LOEWENSTEIN, W., NAKAS, M., SOCOLAR, S. J.: Junctional membrane uncoupling. Permeability transformation at a cell membrane junction. J. gen. Physiol. **50**, 1865–1891 (1967).

LÜTTGAU, H. C., NIEDERGERKE, R.: The antagonism between Ca and Na ions on the frog's heart. J. Physiol. (Lond.) **143**, 486–505 (1958).

LUXORO, M., YANEZ, E.: Permeability of the giant axon of *Dosidicus gigas* to calcium ions. J. gen. Physiol. **51**, 115s–122s (1968).

MARTIN, D. L., DE LUCA, H. F.: Influence of sodium on calcium transport by the rat small intestine. Amer. J. Physiol. **216**, 1351–1359 (1969).

MARTONOSI, A., FERETOS, R.: Observations on the Ca uptake by fragmented sarcoplasmic reticulum. Fed. Proc. **22**, 352 (1963).

MARTONOSI, A., FERETOS, R.: Sarcoplasmic reticulum. I. The uptake of Ca^{++} by sarcoplasmic reticulum fragments. J. biol. Chem. **239**, 648–658 (1964).

MASON, D. T., BRAUNWALD, E.: Studies on digitalis. X. Effects of ouabain on forearm vascular resistance and venous tone in normal subjects and in patients in heart failure. J. clin. Invest. **43**, 532–543 (1964).

MCLAUGHLIN, S. G. A., HINKE, J. A. M.: Sodium and water binding in single striated muscle fibers of the giant barnacle. Canad. J. Physiol. Pharmacol. **44**, 837–848 (1966).

MCLEAN, F. C., HASTINGS, A. B.: Clinical estimation and significance of calcium-ion concentrations in the blood. Amer. J. med. Sci. **189**, 601–613 (1935).

MEECH, R.: Intracellular calcium injection causes increased potassium conductance in *Aplysia* neurones. Comp. Biochem. Physiol. **42A**, 493–499 (1972).

MOREL, F., ROINEL, N., LE GRIMELLEC, C.: Electron probe analysis of tubular fluid composition. Nephron **6**, 350–364 (1969).

MUCHNIK, S., VENOSA, R. A.: Role of sodium ions in response of the frequency of miniature end-plate potentials to osmotic changes in the neuromuscular junction. Nature (Lond.) **222**, 169–171 (1969).

MÜLLER, P.: Ouabain effects on cardiac contraction, action potential and cellular potassium. Circulat. Res. **17**, 46–56 (1965).

MULLINS, L. J., BRINLEY, F. J., JR.: Some factors influencing sodium extrusion by internally dialyzed squid axons. J. gen. Physiol. **50**, 2333–2355 (1967).

MULLINS, L. J., BRINLEY, F. J., JR.: Potassium fluxes in dialyzed squid axons. J. gen. Physiol. **53**, 704–740 (1969).

NICHOLS, G., JR., WASSERMAN, R. H. (Eds.): Cellular Mechanisms for Calcium Transfer and Homeostasis. New York: Academic Press 1971.

NIEDERGERKE, R.: Calcium and the activation of concentration. Experientia (Basel) **15**, 128–130 (1959).

NIEDERGERKE, R.: Movements of Ca in frog heart ventricles at rest and during contractures. J. Physiol. (Lond.) **167**, 515–550 (1963).

NIEDERGERKE, R., HARRIS, E. J.: Accumulation of calcium (or strontium) under conditions of increasing contractility. Nature (Lond.) **179**, 1068–1069 (1957).

NIEDERGERKE, R., LÜTTGAU, H. C.: Calcium and the contraction of the heart. Nature (Lond.) **179**, 1066–1067 (1957).

NEUMAN, W. F., NEUMAN, M. W.: The chemical dynamics of bone mineral, p. 1–22. Chicago: The University of Chicago Press 1958.

OGAWA, Y.: The apparent binding constant of glycoletherdiaminetetraacetic acid for calcium at neutral pH. J. Biochem. (Tokyo) **64**, 255–257 (1966).

OHTA, M., NARSAHASHI, T., KEELER, R. F.: Effects of veratrum alkaloids on membrane potential and conductance of squid crayfish and giant axons. J. Pharmacol. exp. Ther. **184**, 143–154 (1973).

OLIVEIRA-CASTRO, G. M., LOEWENSTEIN, W. R.: Junctional membrane permeability. Effects of divalent cations. J. Membr. Biol. **5**, 51–77 (1971).

ORENTLICHER, M., ORNSTEIN, R. S.: Influence of eternal cations on caffeine-induced tension: Calcium extrusion in crayfish muscle. J. Membr. Biol. **5**, 319–333 (1971).

OSA, T.: Effect of removing the external sodium on the electrical and mechanical activities of the pregnant mouse myometrium. Jap. J. Physiol. **21**, 607–625 (1971).

PAUPE, J.: Comparison entre fractions calciques de liquides cephalo-rachidiens et de serums normaux chez l'Homme. C.R. Soc. Biol. (Paris) **151**, 318–320 (1957).

PORTZEHL, H., CALDWELL, P. C., RÜEGG, J. C.: The dependence of contraction and relaxation of muscle fibres from the crab, *Maia squinado*, on the internal concentrations of free calcium ions. Biochim. biophys. Acta (Amst.) **79**, 581–591 (1964).

POTASHNER, S. J., JOHNSTONE, R. M.: Cation gradients, ATP and amino acid accumulation in Ehrlich ascites cells. Biochim. biophys. Acta (Amst.) **233**, 91–103 (1971).

PRESSMAN, B. C.: Ionophorous antibiotics as models for biological transport. Fed. Proc. **27**, 1283–1288 (1968).

REITER, M.: Cardioactive steroids with special reference to calcium. In: CUTHBERT, A. W. (Ed.): Calcium and Cell Function, p. 270–279. London: Macmillan 1970.

REUTER, H.: Divalent ions as charge carriers in excitable membranes. Progr. Biophys. molec. Biol. **26**, 1–43 (1973).

REUTER, H., BLAUSTEIN, M. P., HAEUSLER, G.: Na–Ca exchange and tension developement in arterial smooth muscle. Phil. Trans. B **265**, 87–94 (1973).

REUTER, H., SEITZ, N.: The dependence of calcium efflux from cardiac muscle on temperature and external ion composition. J. Physiol. (Lond.) **195**, 451–470 (1968).

RINGER, S.: A further contribution regarding the influence of the different constituents of the blood on the contraction of the heart. J. Physiol. (Lond.) **4**, 29–42 (1883).

ROBINSON, R. A., STOKES, R. H.: Electrolyte Solutions. Second edition, revised. London: Butterworths 1968.

RODRIGUEZ DE LORES ARNAIZ, G., DE ROBERTIS, E.: Properties of the isolated nerve endings. Curr. Topics Membr. Transp. **3**, 237–272 (1972).

ROJAS, E., HIDALGO, C.: Effect of temperature and metabolic inhibitors on ^{45}Ca outflow from squid giant axons. Biochim. biophys. Acta (Amst.) **163**, 550–556 (1968).

ROMERO, P. J., WHITTAM, R.: The control by internal calcium of membrane permeability to sodium and potassium. J. Physiol. (Lond.) **214**, 481–507 (1971).

ROSE, R. C., SCHULTZ, S. G.: Studies on the electrical potential profile across rabbit ileum. Effects of sugars and amino acids on transmural and transmucosal P.D.s. J. gen. Physiol. **57**, 639–663 (1971).

ROSENBERG, T., WILBRANDT, W.: Uphill transport induced by counter-flow. J. gen. Physiol. **41**, 289–296 (1957).

ROSSI, C. S., LEHNINGER, A. L.: Stoichiometry of respiratory stimulation, accumulation of Ca^{++} and phosphate, and oxidative phosphorylation in rat liver mitochondria. J. biol. Chem. **239**, 3971–3980 (1964).

RUBIN, R. P.: The metabolic requirements for catecholamine release from the adrenal medulla. J. Physiol. (Lond.) **202**, 197–209 (1969).

RUBIN, R. P.: The role of calcium in the release of neurotransmitter substances and hormones. Pharmacol. Rev. **22**, 389–428 (1970).

RUSSELL, J. M., BLAUSTEIN, M. P.: Calcium efflux from barnacle muscle fibers: dependence on external cations. J. gen. Physiol. **63**, 144–167 (1974).

SCHACHTER, D.: Toward a molecular description of active transport. In: DOWBEN, R. M. (Ed.): Biological Membranes, p. 157–176. Boston: Little, Brown 1969.

SCHACHTER, D., KOWARSKI, S., REID, P.: Active transport of calcium by intestine: studies with a calcium activity electrode. In: CUTHBERT, A. W. (Ed.): Calcium and Cellular Function, p. 108–123. London: Macmillan 1970.

SCHAECHTELIN, G.: Der Einfluß von Calcium und Natrium auf die Kontraktur des M. rectus abdominis. Pflügers Arch. ges. Physiol. **273**, 164–181 (1961).

SCHAFER, J. A., HEINZ, E.: The effect of reversal of Na^+ and K^+ electrochemical potential gradients on the active transport of amino acids in Ehrlich ascites tumor cells. Biochim. biophys. Acta (Amst.) **249**, 15–33 (1971).

SCHATZMANN, H. J.: Herzglykoside als Hemmstoffe für den aktiven Kalium- und Natriumtransport durch die Erythrocytenmembran. Helv. physiol. pharmacol. Acta **11**, 346–354 (1953).

SCHATZMANN, H. J., VINCENZI, F. F.: Calcium movements across the membrane of human red cells. J. Physiol. (Lond.) **201**, 369–395 (1969).

SCHOFFENIELS, E.: Distribution du calcium et du magnesium entre la cellule et son milieu. In: Handbuch der exp. Pharmakol., 17. Bd., 1. Teil, S. 114–130. Berlin-Göttingen-Heidelberg: Springer 1963.

SCHOFFENIELS, E.: Ionic composition of the arterial wall. Angiology **6**, 65–88 (1969).

SCHULTZ, S. G., CURRAN, P. F.: Coupled transport of sodium and organic solutes. Physiol. Rev. **50**, 637–718 (1970).

SCHWARTZENBACH, G., SENN, H., ANDEREGG, G.: Komplexone XXIX. Ein großer Chelateffekt besonderer Art. Helv. chim. Acta **40**, 1886–1900 (1957).

SHANES, A. M.: Electrochemical aspects of physiological and pharmacological action in excitable cells: Part I, The resting cell and its alterations by extrinsic factors. Pharmacol. Rev. **10**, 59–164 (1958a).

SHANES, A. M.: Electrochemical aspects of physiological and pharmacological action in excitable cells: Part II, The action potential and excitation. Pharmacol. Rev. **10**, 165–273 (1958b).

SHATKAY, A.: Individual activity of calcium ions in pure solutions of $CaCl_2$ and in mixtures. Biophys. J. **8**, 912–919 (1968).

SHPORER, M., CIVAN, M. M.: Nuclear magnetic resonance of sodium-23 linoleate-water. Basis for an alternative interpretation of sodium-23 spectra within cells. Biophys. J. **12**, 114–122 (1972).

SILLEN, L. G.: Stability Constants of Metal-Ion Complexes. Section I: Inorganic Ligands, p. 235–236. London: The Chemical Society 1964.

SITRIN, M. D., BOHR, D. F.: Ca and Na interactions in vascular smooth muscle contraction. Amer. J. Physiol. **220**, 1124–1128 (1971).

SMITH, M. W.: The *in vitro* absorption of water and solutes from the intestine of goldfish, *Carassius auratus*. J. Physiol. (Lond.) **175**, 38–49 (1964).

SOMLYO, A. P., SOMLYO, A. V.: Vascular smooth muscle I. Normal structure, pathology, biochemistry and biophysics. Pharmacol. Rev. **20**, 197–272 (1968).

STAHL, W. L., SWANSON, P. D.: Uptake of calcium by subcellular fractions isolated from ouabain-treated cerebral tissues. J. Neurochem. **16**, 1553–1563 (1969).

STAHL, W. L., SWANSON, P. D.: Calcium movements in brain slices in low sodium or calcium media. J. Neurochem. **19**, 2395–2407 (1972).

TEREPKA, A. R., STEWART, M. E., MERKEL, N.: Transport functions of the chick corio-allantoic membrane. II. Active calcium transport, *in vitro*. Exp. Cell Res. **58**, 107–117 (1969).

TOMITA, T., WATANABE, H.: Factors controlling myogenic activity in smooth muscle. Phil. Trans. B **265**, 73–85 (1973).

TOWER, D. B.: Ouabain and the distribution of calcium and magnesium in cerebral tissues *in vitro*. Exp. Brain Res. **6**, 273–283 (1968).

TRINER, L., KILLIAN, P., NAHAS, G. G.: Ouabain hypoglycemia: Insulin mediation. Science **162**, 560–561 (1968).

VAN BREEMEN, C., FARINAS, B. R., GERBA, P., MCNAUGHTON, E. D.: Excitation-contraction coupling in rabbit aorta studied by the lanthanum method for measuring cellular calcium influx. Circulat. Res. **30**, 44–54 (1973).

VAN BREEMEN, C., MCNAUGHTON, E. D.: The separation of cell membrane calcium transport from extracellular calcium exchange in vascular smooth muscle. Biochem. biophys. Res. Commun. **39**, 567–574 (1970).

VAN ROSSUM, G. D. V.: Net movements of calcium and magnesium in slices of rat liver. J. gen. Physiol. **55**, 18–32 (1970).

VASSORT, G.: Influence of sodium ions on the regulation of frog myocardial contractility. Pflügers Arch. ges. Physiol. **339**, 225–240 (1973).

VOGEL, D., BRINLEY, F. J., JR.: Mg and Ca fluxes in isolated dialyzed barnacle muscle fibers. Biophys. Soc. Abstr. Seventeenth Ann. Meeting, p. 104a (1973).

VOGEL, G., STOECKERT, I.: Die Bedeutung des Natriums für den renaltubulären Calcium-Transport bei *Rana ridibunda*. Pflügers Arch. ges. Physiol. **298**, 23–30 (1967).

VOGEL, G., TERVOOREN, U.: Die Bedeutung von Kalium für die renal-tubulären Transporte von Natrium und Calcium und für die Wirkung kardiotoner Steroide. Pflügers Arch. ges. Physiol. **284**, 103–107 (1965).

WALSER, M.: Ion Association. VI. Interactions between calcium, magnesium, inorganic phosphate, citrate and protein in normal human plasma. J. clin. Invest. **40**, 723–730 (1961).

WALSER, M.: Calcium clearance as a function of sodium clearance in the dog. Amer. J. Physiol. **200**, 1099–1104 (1961).

WALSER, M.: Calcium-sodium interdependence in renal transport. In: FISHER, J. W. (Ed.): Renal Pharmacology, p. 21–41. New York: Appleton-Century-Crofts 1971.

WASSERMAN, R. H. (Ed.): The Transfer of Calcium and Strontium Across Biological Membranes. New York: Academic Press 1963.

WASSERMAN, R. H. (Ed.): Calcium Transport by the intestine: A model and comment on vitamin D action. Calcif. Tiss. Res. **2**, 301–313 (1968).

WEBER, A.: Energized calcium transport and relaxing factor. Curr. Top. Bioenerget. **1**, 203–254 (1966).

WEBER, A.: The mechanism of the action of caffeine on sarcoplasmic reticulum. J. gen. Physiol. **52**, 760–772 (1968).

WEBER, A., HERZ, R.: The relationship between caffeine contracture of intact muscle and the effect of caffeine on reticulum. J. gen. Physiol. **52**, 750–759 (1968).

WILBRANDT, W., KOLLER, H.: Die Calciumwirkung am Froschherzen als Funktion des Ionengleichgewichts zwischen Zellmembran und Umgebung. Helv. physiol. pharmacol. Acta **6**, 208–221 (1948).

WILBRANDT, W., ROSENBERG, T.: The concept of carrier transport and its corollaries in pharmacology. Pharmacol. Rev. **13**, 109–183 (1961).

WINEGRAD, S.: Studies of cardiac muscle with a high permeability to calcium produced by treatment with ethylenediaminetatraacetic acid. J. gen. Physiol. **58**, 71–93 (1971).

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The Natural History of Amine Oxidases

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Contents

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I. Introduction

There are many reasons that make the study of a family of enzymes like that of the amine oxidases rewarding. There is, first of all, the elucidation of the *biochemical* properties of these enzymes. This has to start with the aim of purifying the individual catalysts and investigating their physical properties, their chemical composition and the stoichiometry of the enzymic reaction as well as their substrates and their inhibitors. But in addition there is the *cytology* of the enzymes, i.e. all the questions relating to their topography in living tissues and their location in relation to other macromolecules. Then there are the *physiological* questions relating to the function of the enzymes in living tissue.

In the study of the amine oxidases all these aspects have been investigated. Answers have been obtained to some of the questions raised, but there is still room for uncertainty and speculation. The present article does not aim at giving a comprehensive review. There have been recent surveys (see COSTA and SANDLER, editors, 1972; TIPTON, 1973). However, some aspects of topical interest have never been fully reviewed. For example, the rôle of amine oxidases in connective tissue has become important only in the last few years. These are among the questions that will be discussed.

One problem that arises in the description of such a family of enzymes is that of nomenclature and classification. Nomenclature will not be discussed in great detail in what follows, but it cannot be entirely ignored. It is clearly unsatisfactory that various authors have used one and the same name for entirely different catalysts, proteins that differ not only in the chemistry of the enzyme proteins and in the chemical nature of their prosthetic groups, but also in their functional significance.

On the other hand, the variety found in living matter is such that the same name is used, and must be used, for compounds differing very slightly in their chemical constitution. This poses a dilemma: clearly one and the same name may cover individual catalysts slightly differing from each other, e.g. in different species or, in the same species, in different organs. This is a difficulty with which taxonomists are very familiar: SIMPSON (1945) distinguishes the "splitters" from the "lumpers".

Enzymes isolated from different species or from different organs often show slight differences in substrate specificity. One assumes that such differences are often due to the substitution of one, or a few, amino acid residues in the enzyme protein. In addition there is the possibility that substrate specificity may differ because the molecular properties of the enzymes in different locations are different, e.g. there may be differences in the states of aggregation of enzyme protomers.

These considerations are important for the monoamine oxidases, enzymes that have been reported to occur in the form of different isoenzymes. Differences in substrate specificity may therefore be interpreted in some instances by assuming that in different locations the different isoenzymes are present in different proportions.

As to nomenclature, a new name is usually helpful when in two different locations the main substrates are different. Ideally, of course, nomenclature should

be linked to physiological significance. However, since in a great number of instances the function of an enzyme is still obscure, its name is often related to a prominent substrate. To some extent, therefore, nomenclature is provisional until the physiological substrate is clearly recognized.

Historical considerations cannot be fully excluded from a discussion of nomenclature. The early studies of the amine oxidases were concerned with the problem of the biological inactivation of amines of high pharmacological activity, substances present in living tissue or others useful in drug therapy.

The reader can be safely referred to earlier reviews on this subject (see BLASCHKO, 1952a). The earliest study is that by SCHMIEDEBERG (1877) who studied amine metabolites, in particular metabolites of benzylamine, before the individual enzymes involved had been characterized.

The story of the amine oxidases begins in the late twenties when within a year Miss HARE (1928) described the enzyme tyramine oxidase and BEST (1929) the enzyme histaminase. In 1937 it was found that the first of these enzymes also acted upon adrenaline and the related monoamines and that it also oxidized the aliphatic monoamines (BLASCHKO et al., 1937a, b; PUGH and QUASTEL, 1937a, b; KOHN, 1937). The catalyst involved, then known as amine oxidase, acted neither on histamine nor on the short-chain aliphatic diamines, e.g. 1,4-diaminobutane (putrescine) and 1,5-diaminopentane (cadaverine). ZELLER found in 1938 that the histaminase of pig kidney also acted upon these two aliphatic diamines. It might be mentioned here that ZELLER's finding, which was not accepted by all observers, has been fully confirmed in recent years when the pig kidney enzyme was successfully crystallized (YAMADA et al., 1967).

The finding, that there was a complementariness in the substrate specificity of amine oxidase and histaminase, led ZELLER (1938) to propose a new nomenclature for the two enzymes. He called amine oxidase "monoamine oxidase" and histaminase "diamine oxidase". In what follows these two enzymes will be designated by the abbreviations MAO and DAO respectively. In order to avoid confusion, it should be emphasised that in this review these abbreviations will be reserved throughout to these two enzymes exclusively. They will not be used to refer to related enzymes with somewhat similar substrate specificities.

The two enzymes known in 1938 were clearly distinguished, not only by their differing substrate specificities, but also by their characteristic inhibitors. The study of the oxidation of adrenaline and the other catecholamines was made possible by the observation that the so-called autoxidation of these compounds could be inhibited by cyanide, whereas the oxidation by MAO was cyanide-insensitive (BLASCHKO et al., 1937a). On the other hand, DAO is reversibly inhibited by cyanide. Similarly, MAO is not inhibited by semicarbazide and other carbonyl reagents; on the other hand all these compounds are strong inhibitors of DAO.

Since 1938 many other inhibitors of MAO have been found. This followed from the original discovery by ZELLER et al. (1955) that iproniazid was an "irreversible" inhibitor of the enzyme. It is not proposed to include a discussion of the MAO inhibitors in this review.

The distinction between MAO and DAO is still valid today. These two enzymes catalyse the same general reaction:

$$R \cdot CH_2 \cdot NH_2 + H_2O + O_2 \rightarrow R \cdot CHO + NH_3 + H_2O_2$$

There are some general differences to be discussed in this review, especially in regard to the effect of substituting the amino group, but the main difference between the individual enzymes lies in the fact that the character of the group R has a profound effect on whether or not a given amine is oxidized. This tells us something about the character of the enzyme-substrate reaction, but it is also of paramount importance in determining the functional significance of the enzyme.

Both these aspects will be discussed in what follows.

II. Oxidases Containing Flavin

A. The Mitochondrial Monoamine Oxidase (MAO)

1. Flavin as a Constituent of the Prosthetic Group

Many reasons have made it likely for a long time that MAO is a flavin enzyme. However the evidence was based mainly on the similarities between the reactions catalysed by MAO and by the mammalian D-amino acid oxidase respectively (RICHTER, 1937; BLASCHKO, 1952a).

An early, more direct, approach to the problem of the prosthetic group was to study the MAO activity in riboflavin deficiency. Miss HAWKINS (1952b) found that the enzymic activity in the liver of riboflavin-deficient rats was reduced. However, when she compared the loss of enzymic activity with that of D-amino acid oxidase activity in the same organ, neither the loss during deficiency nor the recovery of enzymic activity upon adding riboflavin to the diet were as pronounced with MAO as they were with the marker enzyme. However, it was pointed out that the experiments could not serve to establish the rôle of flavin, since also with the D-amino oxidase no significant fall was seen in the enzymic activity in the kidneys. Somewhat more marked reductions in liver MAO, and more marked recoveries after administering riboflavin, occurred when accumulation of fat was prevented by adding inositol to the diet.

The observations on the rat kidney illustrate the inherent limitations of the method: nobody doubts that D-amino acid oxidase is a flavin enzyme, but in the kidneys the enzyme hangs on to the cofactor even in a severe deficiency. An analogous interpretation was given to HAWKINS' experiments by SOURKES (1968) who repeated and confirmed them. In the meantime, experiments on partly purified preparations of MAO had been carried out which supported the idea that MAO was a flavin enzyme. The experiments from SOURKES' laboratory have recently been summarized (YOUDIM and SOURKES, 1972).

Progress with purification of monoamine oxidase has produced evidence from many authors, indicating that MAO contains flavin adenine nucleotide (FAD).

Work from a number of different laboratories produced evidence of the presence of flavin in the highly purified preparation of the oxidase. ERWIN and HELLERMAN (1967) achieved a considerable degree of purification of the oxidase of mitochondria from the bovine kidney, using digitonin treatment, followed by ammonium sulphate fractionation and column chromatography on calcium phosphate gel-cellulose. The purified preparation had a spectrum characteristic of a flavoprotein, and there was a maximum reduction of absorption at 460 nm, when substrate (benzylamine) was added under anaerobic conditions. Also, the preparation emitted a fluorescence with a peak at 520 nm when the activating wavelength was at 450 nm; this also is characteristic of a flavin compound.

Several other investigators have reported analogous observations. Beef liver mitochondrial MAO was studied by IGAUE et al. (1967), with similar results; this work has recently been reviewed by YASUNOBU and OI (1972). Two chief components, of molecular weights of about 425000 and 1200000 respectively, were isolated, and the FAD content was 1 mole per 100000 g of protein. Similar results were obtained with rat liver oxidase (see SOURKES, 1968; YOUDIM and SOURKES, 1972).

The pig liver enzyme was studied by ORELAND and OLIVECRONA (1971; see also ORELAND, 1971; 1972). Mitochondria served as the starting material, and these were treated with methyl ethyl ketone. Two peaks of activity, with not significantly differing substrate specificity, were eventually isolated, of a molecular weight estimated at 110000 and 280000 respectively. Here again evidence of the presence of flavin was obtained, with an absorption peak at 410 nm and a shoulder with a maximum at 455 nm; the latter showed a decrease when substrate (benzylamine) was added under anaerobic conditions. ORELAND concludes that the flavin is held firmly, by covalent links; this is similar to the conclusion arrived at by YASUNOBU et al. (1968) and by SOURKES (1968).

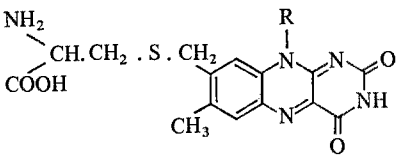
TIPTON (1968a, b) has made a study of the mitochondrial MAO from pig brain. No detergents were used in the purification of the oxidase. The final product, applied to a calibrated column of Sephadex G 200, was found to have a molecular weight of 435000. It was possible to obtain a low-molecular-weight fraction that activated D-amino acid apo-oxidase. One mole of FAD per 120000 g of protein was found. The spectral properties of the enzyme were similar to those described by other authors. An apo-enzyme of MAO was obtained by careful treatment with acid; this material could be re-activated by adding FAD.

The observations just reported raise the question whether in different locations the mode of attachment of the flavin moiety to the enzyme protein is different. It is difficult to envisage that the apo-enzyme of the pig brain enzyme could be able to attach the FAD in covalent linkage once the natural co-factor had been removed. It would be of interest to establish whether or not the re-constituted enzyme is in all respects similar to the native, undissociated enzyme.

The mode of attachment of the flavin moiety to the enzyme protein has recently been studied by KEARNEY et al. (1971) and by WALKER et al. (1971). Advantage was taken of the fact, to be more fully discussed below, that MAO is located in the outer mitochondrial membrane. This allowed separation of MAO from succinic dehydrogenase, another flavin enzyme, in which FAD is covalently linked to the enzyme protein, through an imidazole nitrogen atom of a histidyl

residue. A preparation of bovine liver MAO was obtained which was about 35 times enriched when compared with fresh mitochondria. The preparation was treated with trichloroacetic acid and, after some further purification, digested with trypsin and chymotrypsin. This treatment yielded a clear yellow supernatant fluid, which contained a flavopeptide in the form of flavin mononucleotide (FMN). The study of the amino acid composition of the peptide moiety showed that it was a pentapeptide, containing serine, glycine and tyrosine in the ratio 1:2:1. Hydrolysis with aminopeptidase revealed the presence of the fifth amino acid residue, which remained attached to the flavin, in the form of cysteinyl-FMN, which is not hydrolysed by aminopeptidase.

The thio-ether of cysteine, attached to the riboflavin in position 8, has been synthesized by GHISLA and HEMMERICH (1971). This compound behaves like the flavin-peptide isolated from bovine mitochondrial MAO (WALKER et al., 1971). The thio-ether has the constitution shown:



where R represents the rest of the FAD in the intact enzyme.

WALKER et al. (1971) show the amino acid sequence of the pentapeptide as follows:

Ser–Gly–Gly–Cys–Tyr
|
flavin

These experiments on liver MAO raise a number of questions. Is the mitochondrial oxidase everywhere a flavin compound of this kind? In the experiments of TIPTON, already described, it seems conceivable that the free FAD was obtained by a cleavage of a covalent bond, but the reactivation of the apoenzyme by added FAD remains to be accounted for. Two possibilities suggest themselves: either the pig brain enzyme differs from the bovine liver enzyme by containing FAD in non-covalent form, or the catalytic system can also be completed by adding free FAD to the enzyme protein.

Observations made on the related microbial oxidases (see II, B) are relevant in regard to this problem.

2. Substrates of MAO

Substrate specificity was one of the main topics of an earlier review (BLASCHKO, 1952a). The observations made in recent years have not only greatly increased the number of known substrates of the enzyme, some of them of great physiological significance, but they have also made it possible to arrive at a more precise formulation of the phenomenon of substrate specificity.

The knowledge, discussed on the preceding pages, that MAO is a flavin enzyme, is of relevance to the question of specificity. We can say that an enzyme of this kind will accept substances as substrates which fulfil two conditions: 1. they must be able to interact with the prosthetic group, and 2. they must be able to interact with, or fit into, the enzyme protein.

In the field of the monoamine oxidases, the enormous variety in substrate and inhibitor specificity that is observed is due to the facts that (a) different enzymes carry different prosthetic groups and that (b) the conditions imposed on substrates by the enzyme proteins display an enormous variety.

As to the requirements of specificity imposed by the prosthetic group, it is obvious that the basic amino group has to interact with the flavin group so that the C–N bond in the substrate can be ruptured. The conditions for this have recently been reviewed by HELLERMAN et al. (1972). The most characteristic feature of MAO which distinguishes it from some of the oxidases to be discussed below, is its ability to accept as substrates not only primary amines. In fact, the interest of physiologists and pharmacologists in the enzyme was first aroused when it was found that adrenaline, a secondary amine, was a substrate (BLASCHKO et al., 1937a, b). At the same time it was shown by RICHTER (1937) and KOHN (1937) that hordenine, the N-dimethylated derivative of tyramine, was a substrate. Since then this fact has repeatedly been confirmed (RANDALL, 1946; SMITH et al., 1962). In the ability to act on dimethylated derivatives MAO differs from another flavin enzyme, D-amino acid oxidase, which will act on N-methyl derivatives but not on N-dimethyl derivatives. For a discussion of possible intermediates in the enzymic reaction see the paper by HELLERMAN et al. (1972). SMITH et al. (1962) have also reported a very slow oxidation of N-oxides; this reaction has not yet been studied with the more highly purified preparations now available.

Of the specificity requirements dictated by the substrate-enzyme protein interaction, that which has been most widely studied is the inability of the short-chain aliphatic diamines like 1,4-diaminobutane (putrescine) and 1,5-diaminopentane (cadaverine) to be oxidized; these amines are without affinity for MAO. We shall see in the following that this property, although common to all types of mammalian MAO investigated, is not an absolute requirement: exceptions to the general rule have been found in insects. This fact demonstrates that although the requirements imposed by the interaction with the prosthetic groups are generally valid, those imposed by interaction with the enzyme protein are variable.

This variability is also well illustrated by the fact, already discovered in 1945 (BLASCHKO and DUTHIE, 1945), that with increasing chain length separating the two amino groups in the polymethylene diamine series, the adverse effect of the second amino group diminishes: for instance, 1,10-diaminodecane and 1,12-diaminododecane are good substrates of the MAO of rabbit liver (see BLASCHKO and HAWKINS, 1950).

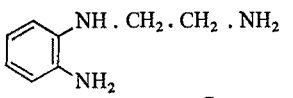
These observations first showed that one requirement for a successful attachment of the substrate to the enzyme was the presence in the substrate of a region free of charged groups and capable of hydrophobic interaction with the enzyme protein. This is a property which has been studied by many contributions from ZELLER'S laboratory.

Another group of compounds studied at Oxford was that of the ω-aminopolymethylene trimethylammonium compounds; in this homologous series

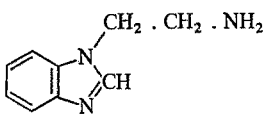
$$H_2N \cdot (CH_2)_n \cdot \overset{+}{N}(CH_3)_3$$

the compounds with $n=5$ or 6 were not significantly oxidized but with increasing chain length the members were substrates and the dodecamethylene derivative was readily oxidized by guinea-pig and rabbit liver preparations albeit less readily than the diamino analogue (BARLOW et al., 1955).

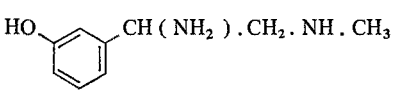
Other diamino compounds tested are the xylylene diamines (BLASCHKO and CHRUSCIEL, 1959). The *meta* compound was rapidly oxidized by both rabbit and guinea-pig liver whereas oxidation was absent or poor with the *ortho* and the *para* derivatives. That the inhibitory effect of the second nitrogen atom in the amino compounds studied is related to the charge on the amino group, is supported by the observation that derivatives of ethylenediamines in which one of the nitrogen atoms is directly attached to an aromatic ring, are substrates of the MAO of rabbit and guinea-pig liver (BLASCHKO et al., 1955). N-*o*-Aminophenylethylenediamine (I) was rapidly oxidized; 1,2′-aminoethylbenziminazole (II) was also attacked. The pKa values of the compound (II) were measured by Dr. ADRIEN ALBERT and found



Compound I



Compound II



Compound III

to be 8.24 and 4.32 respectively. This shows that the terminal amino group is about 90 per cent ionized at the pH of 7.4 whereas the second has extremely weak basic properties. It might be mentioned that LEHMANN and RANDALL (1948) have reported that certain ethylenediamine derivatives are oxidized by the MAO of guinea-pig liver. For instance, Compound (III), the amino analogue of phenylephrine, is oxidized at about the same rate as the latter. This observation appears to merit a more detailed study.

Interference with attachment of the amines to the mammalian MAO is by no means restricted to compounds in which that interference is due to the presence of a positively charged amino (or trimethylammonium) group: the short-chain ali-

phatic ω-amino carboxylic acids, e.g. ω-amino-enanthic acid, $H_2N(CH_2)_6 \cdot COOH$, or ω-amino-caprylic acid, $H_2N(CH_2)_7 \cdot COOH$, are not or poorly oxidized. On the other hand, ω-amino-hendecanoic acid (with 11 carbon atoms) is rapidly oxidized by guinea-pig liver. In other words, here too it is the interatomic distance between the carboxyl and the amino group that determines affinity (BLASCHKO et al., 1962).

A discussion of the physiology of MAO would not be complete without a discussion of its naturally occurring substrates. In 1952 these were still imperfectly known although even 25 years ago it was clear that MAO was not the only catalyst of the inactivation of the catecholamines (see BLASCHKO, 1952a). However, it is the events following the discovery by ARMSTRONG, MACMILLAN and SHAW (1957) of vanillylmandelic acid as a normal metabolite in human urine and the subsequent work by AXELROD (see AXELROD, 1959) on catechol-O-methyl transferase which led to a better understanding of the rôle played by MAO in the catabolism of the catecholamines. In addition, this work led to the recognition of three new and important substrates of MAO: metanephrine, normetanephrine and 3-methoxytyramine.

In addition to these major metabolites it seems likely that both tyramine and octopamine are normally occurring substrates of MAO in mammals. These two monophenolic amines occur in mammals in small amounts; they are however present in large amounts in both molluscs and arthropods (HENZE, 1913; ERSPAMER, 1952; LAKE et al., 1970; BARKER et al., 1972).

The other important substrate of MAO in mammals is 5-hydroxytryptamine (FREYBURGER et al., 1952; BLASCHKO, 1952b). It was the discovery of both 5-hydroxyindoleacetic acid (see UDENFRIEND, 1958) and of 5-hydroxytryptophol (KVEDER et al., 1962) in mammalian urine which showed that MAO is the chief catalyst of 5-hydroxytryptamine breakdown (see BLASCHKO and LEVINE, 1965).

The catecholamines (and their O-methylated metabolites) as well as 5-hydroxytryptamine yield, as a result of the activity of MAO, the corresponding aldehyde. This aldehyde is never the end product of amine breakdown. The first oxidation product is either further oxidized by an aldehyde dehydrogenase to the corresponding carboxylic acid, or it is reduced, by an alcohol dehydrogenase, to the corresponding alcohol.

3. Diversity of Monoamine Oxidases

a) Isoenzymes of MAO

That the relative activities of an enzyme against a variety of substrates differ according to the source of enzyme is a phenomenon that is familiar to students of many enzymes. Some relevant observations will be discussed in some detail below.

There have also been reports that MAO from one organ is not homogeneous but is composed of a number of isoenzymes. These observations will not be discussed here in great detail since little fresh material has accumulated since they were last comprehensively reviewed (COSTA and SANDLER, editors, 1972; SANDLER and YOUDIM, 1972). The problem has been approached in several different ways.

Inhibition studies have shown that some inhibitors will, at a given concentration, produce different degrees of inhibition with different substrates. When the relation between percentage inhibition and inhibitor concentration was studied, it was found that with some inhibitors there was a range in which increasing concentration did not produce a higher percentage inhibition; beyond that range a further increase in inhibition occurred. With different substrates the percentage inhibition achievied at the point of levelling out was different.

One of the first of these inhibitor studies was that of JOHNSTON (1968) who used preparation of rat brain mitochondrial MAO. Using clorgyline as inhibitor, he found a plateau when tyramine was used as substrate. He interpreted his observations by assuming the presence of an enzyme A that was highly sensitive to the inhibitor, with a pI50 value of 8.8, and of an enzyme B that was relatively insensitive to clorgyline, with a pI50 of 5.2. The observations suggested that 5-HT was oxidized by enzyme A only.

Many other authors have described analogous observations, in which similar differences in sensitivity to enzyme inhibitors have been described. Since these studies have been fully reviewed quite recently, the reader is referred to reviews on the subject (TIPTON, in print; GORKIN, 1972; YOUDIM, 1972; NEFF and GORIDIS, 1972; FULLER, 1972; SQUIRES, 1972).

The second set of observations on isoenzymes is based on separation, usually by electrophoresis, of a number of enzymically active entities which migrate differently in the separation. In some instances the pattern of substrate specificity of the separated fractions was essentially similar, in others it was different. Of these latter instances the most significant is a report on the separation of an oxidase that oxidizes specifically, or almost specifically, dopamine (see TIPTON, 1972; YOUDIM, 1972).

Against the existence of isoenzymes it has been argued that MAO has never been obtained entirely pure and separated from contamination with other membrane constituents, in particular phospholipid. The idea has been discussed that the different fractions separated by electrophoresis might represent enzyme fractions that differ from each other in having different kinds of membrane fragments attached (see TIPTON, in press; HOUSLAY and TIPTON, 1973).

It is true that there are differences in the electrophoretic behaviour of enzyme samples, according to whether these are prepared from mitochondria or "microsomes" (see KIM and D'IORIO, 1968). This could well be interpreted as due to an admixture of different membrane fragments to the enzyme. However, it is more difficult to interpret the observations on differential inhibition of enzyme in these terms. It seems best to leave the final resolution of the question of isoenzymes of MAO to results of further experimentation.

A new approach to the problem of multiple forms makes use of immunological techniques. This requires the use of purified enzyme, for the preparation of antibody. GOMES et al. (1969) obtained two fairly highly purified MAO preparations, C1 and C2, from bovine liver mitochondria; these had molecular weights of 400000 and 1200000 respectively, but the same protein: flavin ratios. Fraction C2 was used as antigen in the rabbit. Antiserum against C2 cross-reacted against both fraction C1 as well as C2, and the evidence suggested that the antigenic composition of the two proteins was identical (HARTMAN et al., 1971). The liver also yielded

another enzymically active fraction, A, which was not obtained pure. This fraction also cross-reacted with the anti-C2 serum. On the basis of these observations HARTMAN et al. (1971) suggest that the enzymically active fractions all represent different molecular forms of the same protein. Using the same antibody, from bovine liver, HIDAKA et al. (1971) obtained evidence to show the immunological identity of the bovine brain mitochondrial MAO with bovine liver MAO. However, more recently, HARTMAN and UDENFRIEND (1972) have isolated from bovine brain an enzyme preparation, representing about 20 per cent of the total brain MAO activity, which does not cross-react with anti-C2 serum. This "brain-specific" type of MAO had an activity with benzylamine as substrate that was about 5 times that of the total brain MAO, when compared with kynuramine as standard. Also, the "brain-specific" type of MAO was less sensitive to pargyline than total brain MAO. Similarly, MCCAULAY and RACKER (1973) have shown that in bovine brain there occur two kinds of MAO. One of these does not act on noradrenaline or 5-HT, but acts on benzylamine; this one entirely cross-reacts with an antibody against the bovine liver MAO. The other one, immunologically distinct, acts on both noradrenaline and 5-HT.

In summary, it seems probable that the immunological methods will serve as useful tools in the characterization of the different types of MAO.

b) Species and Organ Diversity of MAO

The proponents of the idea of isoenzymes can point to many observations on differences in substrate specificity determined by the source from which the enzyme was obtained. These instances are not uncommon, and a few will be discussed in what follows.

Species differences have been known for some time. For instance, from early observations by KOHN (1937) and RANDALL (1946) one gains the impression that MAO from pig and cat acts on tertiary amines more readily than enzyme from other mammalian species.

In the following three relatively recent studies from the author's laboratory will be discussed which illustrate the phenomenon of substrate specificity.

α) *Cephalopod MAO.* MAO is present in many species of molluscs (BLASCHKO et al., 1937b; BLASCHKO, 1941; BLASCHKO and HOPE, 1957). Particularly in cephalopods the enzyme has been found in many tissues (BLASCHKO and HAWKINS, 1952; BLASCHKO and HIMMS, 1954). In all these studies slight differences only between these enzymes and mammalian preparations were noted.

However, more recently BOADLE (1967; 1969) has made a study of the substrate specificity of the enzyme in *Eledone cirrhosa*, in which some differences were uncovered. This species contains in many organs an enzyme that acts on amines which are substrates of the mammalian MAO. The aliphatic diamines, putrescine and cadaverine, were not oxidized. A difference was found with heptamethylene diamine, which was oxidized by homogenates of renal appendages, but not of liver. However, the most marked difference was seen in the oxidation of histamine. Extracts of liver or posterior salivary glands did not act on histamine but those of

pancreas, the renal appendages and the optic ganglia oxidized histamine. That this oxidation was due to MAO was supported by these observations:

1. Iproniazid and harmine inhibited the oxidation of histamine by the renal appendages extract.

2. Mixed-substrate experiments, using histamine and N-methyl β-phenylethylamine established that oxygen uptake with the two amines was competitive and not additive.

3. The preparations acting on histamine also acted on ω-N-methyl histamine, a substance not acted upon by mammalian histaminase.

These observations established that in some of these tissues there occurred an oxidation of histamine which was brought about by MAO, the first report on a "histaminase" type of reaction in an invertebrate. From a study of the relationship between the relative rates of oxidation and pH it seems that the *Eledone* enzyme accepts the monocationic form of histamine in which the imidazole ring does not carry a charge.

These observations are of more than theoretic interest: some time ago, BERTACCINI (1961) reported that in *Eledone moschata* treatment with a number of MAO inhibitors *in vivo* raised the histamine content of the optic ganglia. Thus, the MAO present controls the histamine concentration in the ganglion. The rapid rise of the histamine content, upon adding the inhibitor, found by BERTACCINI indicates a very rapid turnover of histamine in the nerve tissue of this species.

β) *Insect MAO.* The first study of MAO in an insect was made on the cockroach, *Periplaneta americana L.* (BLASCHKO et al., 1961). Feeble enzyme activity was found in a number of tissues, but in homogenates of Malpighian tubules the MAO activity was of an order of magnitude quite comparable to that found in the mammalian liver. In this early study nothing was found that distinguished the enzyme from mammalian MAO: it acted on β-phenylethylamine and on tryptamine; it also acted on N-methylated amines.

It was only when the preparations from the Malpighian tubules were subjected to a closer scrutiny (BOADLE, 1967; BOADLE and BLASCHKO, 1968), that several distinctive properties of the insect enzyme came to light; it was found that the preparation also acted upon agmatine, the product of decarboxylation of arginine, and upon some of the short-chain aliphatic diamines, in particular cadaverine (1,5-diaminopentane) and 1,7-diaminoheptane. The latter amine was attacked most rapidly.

The Malpighian tubules of another species of cockroach were also examined for enzymic activity. This was *Blaberus discoidalis.* No oxidation of any of the "conventional" substrates of MAO was seen, but the *Blaberus* preparation acted on agmatine and upon the aliphatic diamines, putrescine, cadaverine, 1,6-diaminohexane and 1,7-diaminoheptane. In other words, here is an oxidase that does, in the manometric experiments, not act on any of the substrates that are commonly considered as related to the physiological substrates of MAO. The *Blaberus* enzyme, in spite of its similarity to the *Periplaneta* enzyme, might be listed as a "diamine oxidase" by some observers. It seems worth while noting, however, that PAU and ACHESON (1968) have reported the presence of 3-hydroxy-4-O-β-D-glucosidobenzyl alcohol in the left colleterial gland of *B. discoidalis;* this might indicate that either dopa or dopamine is deaminated in this species.

For reports on insect MAO see also CHAUDHARY et al. (1967), SHAMBOUGH (1969), WHITEHEAD (1970), HAYES et al. (1971, 1972).

γ) *Cyclostome MAO.* A study was made of the substrates of amine oxidase(s) in these animals (BOADLE, 1967; BLASCHKO et al., 1969), because they represent the most primitive of the extant vertebrates. Although this study must be considered as a preliminary one it has yielded several results of interest for the student of substrate specificity.

YOUNG (1962), whose nomenclature has been followed, distinguishes in the superorder *Agnatha* two orders; members of both of these orders have been tested.

The order *Myxinoidea* includes *Myxine glutinosa L.*, the hagfish. The liver of this species contained an active MAO, which in its pattern of substrate specificity was similar to that known for mammalian MAO. The amines that were oxidized were those acted upon by the mammalian MAO; compounds like histamine, agmatine or the short-chain aliphatic diamines were not oxidized. A similar type of activity was present in the gills, the kidneys and the heart.

The second order are the *Petromyzontia. Petromyzon marinus L.*, the sea lamprey, yielded preparations that revealed activities markedly different from the mammalian pattern. The liver of *P. marinus* (taken from adult specimens, caught in the tidal reaches of the river Severn) oxidized a great variety of substrates, including many compounds that are not substrates of mammalian MAO. Histamine was extremely rapidly oxidized. Agmatine was also attacked. A number of amines found to be oxidized by the *P. marinus* liver extracts are listed in Table 1. The Table shows that putrescine was rapidly oxidized by *P. marinus* liver. The Table does not include the other aliphatic diamines of the homologous series $H_2N(CH_2)_nNH_2$, but Fig. 1 shows that many of them were oxidized; the rate of oxidation of putrescine ($n=4$) and 1,6-diaminohexane was particularly high but there was another peak at $n=13$.

Experiments with *P. marinus* kidney revealed a striking difference in the range of substrates attacked by preparations from the two organs. With the kidney preparation, the rates of oxidation of histamine, putrescine, *n*-heptylamine and γ-phenylpropylamine were not significant. The amines most rapidly oxidized by the kidney preparation were tryptamine, ω-N-methyltryptamine, 5-methoxytryptamine, 5-hydroxytryptamine, N-methyl-β-phenylethylamine and β-phenylethylamine.

The remarkable range of substrates that is oxidized by the sea lamprey liver preparation raises the question as to whether this tissue contains a true histaminase (DAO). This possibility has not been fully ruled out but it is unlikely on account of the following observations: 1. acetone powders of *P. marinus* liver retained the ability to act on histamine in the insoluble residue, the fraction that contains the MAO activity; histaminase is usually recovered in the eluates of such powders; 2. the oxidation of histamine and *n*-heptylamine, a typical MAO substrate, was competitive and not additive.

Another member of the order *Petromyzontia*, the river lamprey, *Lampetra fluviatilis L.*, was already studied by HOPE (1956). These early observations which showed the presence of MAO activity have been confirmed and extended (BOADLE, 1967). The preparations of *L. fluviatilis* liver acted rapidly on histamine, putrescine and agmatine as well as on typical substrates of MAO. Extracts of heart muscle and of gastrointestinal tract oxidized tryptamine, its ω-N-methylated derivative as well as β-phenylethylamine; they were without action on histamine or putrescine.

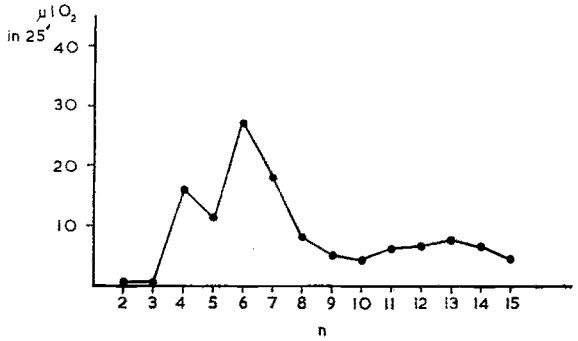


Fig. 1. Rates of oxidation of the members of the homologous series of aliphatic diamines, $H_2N \cdot (CH_2)_nNH_2$, by a dialysed homogenate of liver from *Petromyzon marinus*. (Unpublished experiments by Dr. S. J. STRICH.) Abscissa: number (n) of carbon atoms in the polymethylene chain. Ordinate: μlO_2 consumed in the first 25 min of the reaction. Each manometer flask contained the equivalent of 310 mg of fresh tissue, in a total volume of 0.7 ml. Initial substrate concentration: 10^{-2} M; $t = 37.5°$ C; gas phase: O_2

Table 1. Relative rates of oxidation of a number of amines by a preparation from *Petromyzon marinus* liver. The rate of oxidation of histamine is taken as 100 (after BOADLE, 1967).

Substrate concentration 10^{-2} M					
methylamine	0	*n*-dodecylamine	10	*m*-xylylenediamine	77
ethylamine	17	*iso*-amylamine	19	*p*-xylylenediamine	67
n-propylamine	17	benzylamine	71	*o*-xylylenediamine	30
n-butylamine	28	*β*-phenylethylamine	56	4-methoxy-*β*-phenylethylamine	128
n-amylamine	30	*γ*-phenylpropylamine	31	3-methoxy-*β*-phenylethylamine	93
n-hexylamine	22	tryptamine	78	3,4-dimethoxy-*β*-phenylethylamine	89
n-heptylamine	33	1,4-methylhistamine	98	2,3-dimethoxy-*β*-phenylethylamine	49
n-octylamine	33	4-picolylamine	97	2,5-dimethoxy-*β*-phenylethylamine	10
n-decylamine	25	3-picolylamine	65	3-methoxy-4-hydroxy-*β*-phenyl-ethylamine	42
n-undecylamine	17	2-picolylamine	46	mescaline	5

These experiments are described in some detail because they show that the amine oxidases of cyclostomes would be a most interesting material for a more systematic study. It can be seen that the two more closely related species which belong to the same order have enzymic activities that are rather similar; also in these two species there is a particularly diversified activity in the liver, indicating the presence of a marked organ specificity. On the other hand, the member of the other order, *Myxine glutinosa*, had an enzyme with a substrate specificity closely similar to that found in other vertebrate species. These observations indicate that the development of amine oxidases in the order *Petromyzontia* followed a course that differs from the main stream of vertebrate evolution, a change that did not happen in the *Myxinoids*.

The diversity of the enzyme in the lampreys, and particularly the organ differences, could be readily interpreted in terms of isoenzymes, but no information on this is as yet available.

δ) Mammalian MAO. Slight differences in substrate specificity do occur in different mammalian species. In addition there are differences within one species, between MAO from different organs. Such observations go back to early days of research on MAO (see ALLES and HEEGAARD, 1943); they were followed up by HAGEN and WEINER (1959) and by HOPE and SMITH (1960). The latter authors described a very impressive difference in the relative rates of oxidation of a number of amines in the organs of the mouse. Choosing 5-hydroxytryptamine (5-HT) as their standard of comparison, they found that in liver homogenates tyramine was oxidized at almost four times the rate of 5-HT, at about 1.5 times in brain and at about 0.8 times that of 5-HT in the kidney. Benzylamine, attacked at about the same rate as 5-HT in the liver, and at about one-quarter the rate for 5-HT in brain, was not acted upon at all in the kidney.

More recently observations of a similar kind have been reported by other authors (see e.g., RAGLAND, 1968).

It is obvious that observations like those just described could also be readily interpreted in terms of the existence of isoenzymes with varying patterns of substrate specificity: the various organs might contain mixtures of the isoenzymes in differing proportions.

Since most of the organs studied are composed of a variety of cell types one might assume that each type contains a different kind of MAO. Elegant demonstrations of such differences were given by GORIDIS and NEFF (1971a; see below) and by JARROTT (1971), following up observations by JARROTT and IVERSEN (1971) on the fall of MAO activity in the vas deferens of a number of species after sympathetic denervation; in these experiments a drop in MAO activity of about 50 per cent occurred, indicating that about one-half of the enzymic activity was located in the adrenergic neurones. JARROTT (1971) found that the relative activities of oxidation of a number of amines in the denervated vas deferens differed from those in the normally innervated tissue, suggesting that the enzyme that disappeared when the nerve supply degenerated was different from the enzyme that remained in the tissue after denervation.

The observation, that in some adrenergically innervated tissues denervation removes enzymic activity, is of interest in connection with early observations from J. H. BURN'S laboratory (see BURN, 1952) in which MAO was compared to acetylcholinesterase at cholinergic nerve endings. Although these observations have been confirmed, the role of MAO, to be discussed later, differs from that assigned to acetylcholinesterase.

The interpretation given by JARROTT and IVERSEN (1971) to their observations cannot be applied to all instances where a fall of MAO occurs after denervation. This is particularly true for organs in which the MAO content is high and where much of the enzyme is located in the parenchymal cells. The salivary glands are such organs, in fact, the human submaxillary and parotid glands have a MAO content higher than that found in any other tissue (STRÖMBLAD, 1959). In the cat's salivary glands, parasympathetic denervation caused a loss of about 50 per cent of the enzymic activity, but this fall was accompanied by a similar fall in the weights

of the glands, so that the activity per unit of weight remained essentially unchanged (STRÖMBLAD, 1956; see also SNYDER et al., 1965). JONASON (1969) estimates the contribution made by the adrenergic innervation to the total MAO content at about 30 per cent, with the remainder located in the parenchyma cells.

As to the cytochemical localization of MAO in salivary glands (see also FUJIWARA et al., 1966).

SNYDER et al. (1965) and HAKANSON and OWMAN (1966) have studiel the loss of MAO in the pineal gland after sympathetic denervation. According to the latter authors only in the cat pineal gland does the bulk of the MAO activity reside in the adrenergic neurones, whereas in the rat and the rabbit gland the enzyme is present mainly in the pinealocytes. Similarly, GORIDIS and NEFF (1971a) estimate that in the rat pineal gland about 6 per cent of the ability to oxidize tyramine is lost after denervation, whereas 70 per cent of the activity against 5-HT as substrate is lost; they interpret these observations in terms of the presence of an isoenzyme, enzyme A (clorgyline-sensitive) in JOHNSTON's (1968) nomenclature, in the adrenergic nerves.

In this connection, it might be mentioned that GORIDIS and NEFF (1971b) have presented evidence in favour of a specific association of JOHNSTON's (1968) isoenzyme type A with adrenergic sympathetic neurones. This type represents about 90 per cent of the total enzyme present in the rat superior cervical ganglion.

It should be pointed out that the relative roles played by the extraneuronal as the intraneuronal MAO in the disposal of the adrenergic mediator cannot be properly assessed by experiments like those just described, which were carried out using tissue homogenates. This is due to the fact that the amount of amine that is admitted to the compartments containing the enzyme depends upon the efficiency of the uptake mechanisms that are at work at the level of the neuronal membrane on the one hand and of the membranes of the tissue cells containing the enzyme on the other (see also TRENDELENBURG, 1972).

An illustration of the part played by the intraneuronal MAO in the disposal of noradrenaline has been obtained in the isolated rabbit ear artery by DE LA LANDE and JELLETT (1971). In this preparation, in which the autonomic nerve endings lie at some distance from the lumen, close to the outer edge of the muscular layer, the response to amine applied intraluminally is not affected by the MAO inhibitor nialamide. On the other hand, the response to noradrenaline applied from the outside is potentiated by nialamide, and the response is not fully terminated immediately after wash-out. This potentiation and prolongation of the response to extraluminally applied noradrenaline is not present in preparations treated with cocaine, an inhibitor of neuronal uptake; nor is it present in sympathetically denervated preparations.

Biochemical and histochemical studies have established that the adrenergic neurones are rich in MAO, but whether this association is specific to this type of neurone is less clear. Also, most measurements of enzymic activity have been made on ganglion cells. It is generally agreed that the sympathetic ganglia are rich in MAO whereas the spinal gnaglia are not (CONSOLO et al., 1968; GIACOBINI and KERPEL-FRONIUS, 1970; TJÄLVE, 1971). However, the nodose ganglion also contains a fair amount of MAO (GIACOBINI and KERPEL-FRONIUS, 1970). The same authors find a relatively low MAO content in the ciliary ganglion of the cat (see also

KOELLE and VALK, 1954; HUIKURI, 1966). FURNESS and COSTA (1971) have not seen any specific association of MAO with adrenergic neurones in the enteric ganglia of the guinea-pig (see also WOHLRAB, 1961; BAXTER-GRILLO, 1970).

What one would most like to know is if there is a specific association of MAO with adrenergic nerve endings, but this has not been established at present (see, however, the observations by MCLEAN and BURNSTOCK, 1972, to be discussed more fully below). The histochemical techniques that have been developed in recent years (BOADLE and BLOOM, 1969; BLOOM et al., 1972; HANKER et al., 1973) should be suitable for the study of this problem. It would be particularly interesting to study the location of MAO in those tissues in which adrenergic and cholinergic autonomic neurones have closely adjoining endings; such an arrangement has been particularly studied in the iris (HÖKFELT, 1966; HÖKFELT and JONSSON, 1966; EHINGER and FALCK, 1966; EHINGER et al., 1970) but it also occurs in other sites (TRANZER and THOENEN, 1967).

For the understanding of the neuronal metabolism of noradrenaline the study of the latter's metabolites may become of interest (see SHARMAN, 1973). In an analysis of the catabolites of noradrenaline in guinea-pig atria ADLER-GRASCHINSKY et al. (1972) have found that changes in the amounts of 3,4-dihydroxyphenylglycol reflected the amounts of noradrenaline that had been oxidized intraneuronally. It seems that the aldehyde formed as the first product of the oxidative deamination of noradrenaline was mainly metabolized by reduction in the neurone, but by further oxidation in the extraneuronal tissues.

4. Intracellular Localization of MAO

All observers have confirmed the early observations on the localization of MAO in the cell. These were carried out on preparations modelled on Warburg's "Körnchensuspension" (see BLASCHKO, 1972). MAO is a preponderantly mitochondrial enzyme (COTZIAS and DOLE, 1951; HAWKINS, 1952a).

It was noticed in this early work on rat liver (HAWKINS, 1952a) that some of the MAO activity was located in the "microsomes". This has been shown to be true also for the MAO of rabbit liver (ZUBRZYCKI and STAUDINGER, 1967). Miss HAWKINS argued that possibly some at least of the microsomal oxidase was in fact of mitochondrial origin (see also BAUDHUIN et al., 1964). This will be more fully discussed below.

We owe a fresh insight into the question of the mitochondrial location of MAO to SCHNAITMAN et al. (1967), who succeeded, by a graded treatment of rat liver mitochondria with digitonin, in obtaining preparations of the outer mitochondrial membranes free of the "mitoplasts", i.e., the inner membranes plus matrix material. The MAO activity was present in the outer membrane fraction. This observation is now generally accepted.

Independent support for these observations comes from electron microscopic studies. Using a tetrazolium salt that gives a formazan capable of reaction with osmium tetroxide (SELIGMAN et al., 1967), BOADLE and BLOOM (1969) studied the location of MAO in the cells lining the proximal convoluted tubules of the guinea-

pig kidney, cells known to be rich in enzyme (BLASCHKO and HELLMANN, 1953). BOADLE and BLOOM demonstrated that the formazan precipitated in the interspace between outer and inner membrane, beginning at the inner surface of the outer membrane, always leaving the interspace between the inner membranes in the cristae infoldings clear (see also BLOOM et al., 1972).

A number of authors, using methods different from that of SCHNAITMAN et al. (1967), have confirmed their results (see BEATTIE, 1968; SOTTOCASA et al., 1967). Use has frequently been made of MAO as a marker enzyme for the outer mitochondrial membrane (see SOTTOCASA, 1967; OKAMOTO et al., 1967; BEATTIE et al., 1967; NEUPERT et al., 1967, 1968; BYGRAVE and BÜCHER, 1968; BRUNNER and NEUPERT, 1968; KAISER and BYGRAVE, 1968; SCHNAITMAN and GREENAWALT, 1968; PEDERSEN and SCHNAITMAN, 1969; BRUNNER and BYGRAVE, 1969).

This new work on the location of MAO is of interest in relation to the question of extramitochondrial MAO. There seems little doubt that the origin of the outer membrane of the mitochondria differs from that of the mitoplast. The question can be asked: at what time is the mitoplast covered by the outer membrane? Is it possibly after the MAO has been incorporated into the outer membrane? If so, it would be easy to understand the nature of the "microsomal" MAO: it could be enzyme located in membrane material still unconnected, or only loosely connected, with the mitochondrion.

Arguments similar to those just given have also been discussed by JARROTT and IVERSEN (1968). These authors also discuss a series of earlier papers in which a "microsomal" location for MAO was described; these need therefore not be referred to again. However, mention should also be made of the observations of WEISSBACH et al. (1957) on "soluble" liver MAO in the guinea-pig's liver. This might be enzyme not yet bound to the outer membrane.

The question as to the origin of mitochondria is still under discussion. It seems to be generally agreed, however, that the outer membrane has its origin outside the mitochondria, and that its proteins are under the control of nuclear deoxyribonucleic acid (see BAXTER, 1971). In other words, it seems likely that any differences in the amounts of MAO and in the substrate specificity of the enzymes in different cells are under the control of the nucleus.

Studies of the regeneration of MAO in rat liver are relevant to the question of the incorporation of MAO into the mitochondria. In observations by HAWKINS and WALKER (1952) it was shown that in the first two days after partial hepatectomy there occurred an increase in liver weight without much increase in total enzyme content. In other words, there occurred a decrease in the activity per unit weight. A similar decrease has since been reported by GEAR (1970), who found that enzymes of the inner and the outer mitochondrial membranes did not regenerate synchronously; in fact, the regeneration of the individual outer membrane enzymes was asynchronous. In this study the recovery of extramitochondrial enzyme was not followed, but this was done in the observations by ERWIN and SIMON (1969), in which the regeneration of enzyme in the rat liver after the application of irreversible inhibitors of MAO (pargyline or iproniazid) was followed. It was found that recovery in the microsomal fraction preceded that in the mitochondria. These observations do suggest that the "microsomal" enzyme is, at least in part, precursor of mitochondrial enzyme.

MAO must be a minor constituent of the outer membrane protein. RACKER and PROCTOR (1970) have used the digitonin method for preparing outer mitochondrial membranes from renal cortex, by subjecting the membranes to a further digitonin treatment. In this way they obtained a preparation of membranes free of MAO and the oxidase in a soluble form. The removal of the enzyme had not diminished the cohesion of the membrane.

When RACKER and PROCTOR (1970) added the soluble enzyme to enzyme-free preparations of outer membrane they noted that the enzyme was again sedimented with the membrane. Does this observation indicate that the membrane contains a specific site to which the enzyme attaches itself? Here is a new problem that is posed by these recent findings. There are indications that phospholipids are involved in the binding. OLIVECRONA and ORELAND (1971), in their attempts to elute MAO from mitochondria, have found that both phosphatidylcholine and phosphatidylethanolamine could be extracted without detaching MAO, but that there was a correlation between the elution of the anionic phospholipids (in particular, cardiolipin) and that of MAO (see also ORELAND, 1972). OLIVECRONA and ORELAND have also observed binding of MAO to pig liver mitochondria freed of enzyme; the MAO was detached by phospholipid elution; when cardiolipin was added subsequently the mitochondria were able to bind MAO.

The author has already once briefly reported on experiments carried out in 1939, on "soluble" preparations of liver MAO obtained by adding lysolecithin to granule suspensions (see BLASCHKO, 1952a). It would be interesting, in the light of recent studies, to know how lysolecithin exerts its solubilizing action.

5. Functional Significance of MAO

The study of MAO has been rewarding for an ever increasing number of investigators because the functional significance of the enzyme in higher animals is clear: MAO is one of the principal catalysts of the biological inactivation of amines normally present in the organism, with the catecholamines and 5-HT as the best studied and most widely distributed substrates. Thus, interest in MAO has grown with the widening recognition of the rôle played by these amines in the animal body.

When catechol-O-methyl transferase was discovered, the emphasis of research for a while veered away from MAO. This phase did not last long. It is true that in many sites oxidative deamination of the catecholamines is preceded by O-methylation but the re-awakening interest in MAO was due to the recognition of the rôle of MAO in adrenergic neurones. The response of excitable organs to catecholamines is terminated by the lowering of amine concentration in the extracellular space. An important factor in lowering extracellular amine concentration is uptake of amine by the endings of adrenergic neurones. MAO is responsible for keeping the concentration of free intraneuronal amine at a low level. This is best demonstrated when the other mechanism that lowers free intraneuronal amine concentration, that is, uptake of amine by the amine-carrying vesicles in the axoplasm, is inhibited, e.g. by reserpine. Under these conditions catecholamine, newly synthetized in the axoplasm or taken up into the axoplasm from the extraneuronal

space, is exposed to the action of intraneuronal MAO. The disappearance of free intraneuronal amine can be prevented by MAO inhibitors (see KOPIN, 1964; CARLSSON, 1966).

Thus, MAO is one part of the mechanism by which the free intraneuronal amine is kept at a low level. For the response of the effector organ, the extracellular amine concentration in the neighbourhood of the receptors on the effector cells is important. This concentration of free amine in the extraneuronal space depends on amine uptake, and it has been shown that this uptake is interfered with when MAO is inhibited. This has recently been fully discussed (TRENDELENBURG, 1972; TRENDELENBURG et al., 1972).

The rôle of MAO in controlling free amine concentration is the basis for the use of MAO inhibitors in mental illness. The antidepressant drugs act by increasing the concentration of free extracellular amine. There are two classes of compounds that have this effect. The first includes the tricyclic antidepressants, e.g. imipramine. These compounds have no significant inhibitory action on MAO; they act directly as inhibitors of catecholamine uptake at the level of the neuronal membrane. The second class of antidepressants are the MAO inhibitors. Their immediate action is to raise the concentration of free intraneuronal amine. They act as inhibitors of catecholamine uptake indirectly, by reducing the gradient of concentration of free amine from extracellular space to the axoplasm.

This difference in the mode of action of the two classes of antidepressant drugs has not always been fully understood. For instance, we read in a recent report on Biochemical Research in Psychiatry (Medical Research Council, 1970): "It has been found that some of the MAO inhibitors, like the tricyclic drugs, prevented re-uptake of catecholamines, and that this property rather than their potency as MAO inhibitors, ran parallel with therapeutic activity." In the light of what has just been discussed it is clear that it is just this inhibition of intraneuronal MAO that makes the compounds like iproniazid inhibitors of neuronal uptake. As a matter of fact, the different sites of attack of the MAO inhibitors on the one side and of the tricyclic drugs on the other may be responsible for the differences that some observers see in the modes of action of these two classes of antidepressant (see PARE, 1972).

Another possible way by which MAO inhibitors might act is by increasing amine output. If the intraneuronal amine is not destroyed by MAO more might be expected to be taken up by the storage organelles. Such an increased amine content could lead to an increased amine release.

Although much is now known as to the fate of the adrenergic mediator, our information on the removal of circulating catecholamine is less complete. Possibly in the disposal of adrenaline the O-methylation is of primary importance. Also, it is known that the efficacy of neuronal uptake decreases with the distance of the receptors from the adrenergic nerve endings (see TRENDELENBURG, 1972).

An additional uncertainty is introduced by our ignorance of the importance of extraneuronal inactivation of amines. That much of the MAO is located in extraneuronal sites has already been discussed. There is the histochemical evidence, some of it already quoted, that in the proximal convoluted tubules of the kidney the epithelial cells contain the enzyme (BLASCHKO and HELLMANN, 1953; GLENNER et al., 1957; BOADLE and BLOOM, 1969). Using the electron-microscopic method of

BOADLE and BLOOM (1969), HORITA and LOWE (1972) have likewise demonstrated the presence of MAO in the myocardial mitochondria.

There is much evidence that shows that extraneuronal uptake of catecholamines occurs (see AVAKIAN and GILLESPIE, 1969; GILLESPIE and MUIR, 1970; GILLESPIE et al., 1970; FISCHER et al., 1965; EISENFELD, 1967a, 1967b; LIGHTMAN and IVERSEN, 1969; DRASKOCZY and TRENDELENBURG, 1970). What is under debate is mainly whether or not uptake into the effector cells is a mechanism by which the response of the cell is terminated (see KALSNER and NICKERSON, 1968; 1969a, b; TRENDELENBURG, 1972).

The biochemist finds it difficult to escape the conclusion that some inactivation at least of catecholamine does occur within the effector cells with adrenergic innervation. It is not known whether or not any of the biological actions of the catecholamines require the entry of amine into the cell. There may be differences between different types of cell in this regard. One of the puzzling features of amine biochemistry is the high amine-forming potential of certain tissues. HOLTZ et al. (1938) discovered L-dopa decarboxylase in the guinea-pig kidney, and the very active enzymic activity of mammalian liver was established soon afterwards (BLASCHKO, 1939). The physiological significance of these findings is still obscure. One suspects that amines in these tissues may have local, intracellular, functions; such a function might account for the presence of a catalyst of amine catabolism. Discussions on catecholamine function have long been dominated by the phenomena of contraction or relaxation of smooth muscle, easily studied by classical pharmacological techniques. The full extent of the rôle of catecholamines in cell metabolism is still to be unravelled. This is a problem not within the scope of the present review.

Another argument in favour of a physiological rôle of MAO in non-neural tissue might be seen in observations on inducibility of enzyme. It has been shown that the enzyme content of tissues is dependent on hormone control.

As an example one can consider the effect of the thyroid hormone on liver MAO. Over twenty years ago SPINKS and BURN (1952) found that the MAO activity of rabbit liver decreased upon feeding of thyroid whereas thyroidectomy caused a rise in the enzymic activities of the rabbit and rat liver enzyme. This observation has been repeatedly confirmed (TRENDELENBURG, 1953; ZILE and LARDY, 1959; ZILE, 1960; HO-VAN-HAP et al., 1967). A more recent study by OKAMOTO (1971) is of particular interest. He isolated the outer mitochondrial membranes of rat liver and studied the activities of three outer membrane enzymes, monoamine oxidase, kynurenine 3-hydroxylase and rotenone-insensitive NADH-cytochrome C reductase in normal and L-thyroxine-treated animals. He found that the specific activities of all these enzymes were decreased to about the same extent. A study of one of these enzymes, kynurenine 3-hydroxylase, suggested that the decrease in enzymic activity was due to a decrease in total enzyme protein rather than to the production of a catalytically less active form of enzyme.

Adrenalectomy also affects MAO in some, but not in all tissues of the rat (AVAKIAN and CALLINGHAM, 1968; CAESAR et al., 1970). In the rat heart a two- to five-fold increase in MAO occurs; this increase can be prevented by administering hydrocortisone to the adrenalectomized animals (CALLINGHAM and DELLA CORTE, 1971). Adrenalectomy seems to exert a general effect on the mitochondrial enzymes

that are located in the outer membrane: NADH-cytochrome C reductase was similarly affected. On the other side, the activities of fumarase and both succinic and malic dehydrogenases were unchanged.

HORITA and LOWE (1972) have recently summarized the observations on an increase of heart MAO in the male rat with age (see also STUDER et al., 1964; MÜLLER and PEARSE, 1965). It has now been found that this increase was also paralleled by a rise in cytochrome C reductase (CALLINGHAM and DELLA CORTE, 1972). Thus, it appears that both age and changes in cortical hormone affect the enzyme content of the outer mitochondrial membrane generally.

HORITA and LOWE (1972) have given reasons for the belief that the effect of age (see NOVICK, 1961) is exerted on the extraneuronal mitochondria. The site of increase in MAO after adrenalectomy may also be extraneuronal, but this is not yet known with certainty. In the vas deferens of the rat an increase by 40 per cent occurred after bilateral adrenalectomy; this increase could be prevented by dexamethasone. It is interesting that this increase did not occur in the sympathetically denervated rat (SAMPATH and CLARKE, 1972). In other words, it is likely that in the vas deferens the hormonal effects are exerted upon the intraneuronal MAO.

The fact that these hormonal changes are effects exerted upon more than one of the outer membrane enzymes illustrates the complexity of the mechanisms of control of enzyme formation. One possibility is that the reason for changes of enzyme content is the variation in enzyme binding sites. It is known that hydrocortisone has an effect upon developing adrenergic structures (ERÄNKÖ and ERÄNKÖ, 1972; ERÄNKÖ et al., 1972; LEMPINEN, 1964): it increases the catecholamine content of the small intensely fluorescing cells of sympathetic ganglia. Here the morphologically demonstrable effect of hydrocortisone must be on the number of amine storage sites. Of course, these small cells are different from the neurones studied by SAMPATH and CLARKE (1972), but it is important to bear in mind that the action of hydrocortisone on adrenergic structures can vary.

It is not intended here to discuss all the effects of hormones on MAO. Some of the varied actions of the sex steroids on the uterus have recently been reviewed (SOUTHGATE, 1972).

The observations discussed in this section leave little doubt that both intraneuronal and extraneuronal MAO have a part to play in the biological inactivation of the catecholamines. As far as extraneuronal function is concerned, differences in extraneuronal uptake of amines in different types of cell may exist which are still little studied.

Another naturally occurring type of amine, not related to the catecholamines, is 5-HT. This amine is also inactivated by MAO. It shares with the catecholamines the property of being formed by, stored in and released from both secretory cells and neurones. In invertebrates, tryptaminergic neurones are not uncommon. In vertebrates these types of neurone have recently been investigated in the central nervous system (see FUXE et al., 1968). Study of the uptake of 5-HT by structures that normally store the amine is complicated by the fact that the uptake mechanisms for catecholamines, uptake 1 and uptake 2 in IVERSEN's (1967) terminology, will be active on 5-HT. In addition, adrenal medulla and adrenergic nerves store 5-HT when the organism is flooded with 5-hydroxytryptophan. Here it is probably chiefly the amino acid that is taken up and decarboxylated intracellularly.

In recent years, the uptake and storage of 5-HT has been repeatedly reviewed (see SHASKAN and SNYDER, 1970; IVERSEN, 1971; CARLSSON et al., 1972).

It is known that MAO is the main catalyst of 5-HT inactivation. The breakdown of 5-HT circulating in the blood stream follows upon its uptake into the blood platelets. These elements do contain MAO (see PAASONEN, 1961; COLLINS and SANDLER, 1971). The rapid uptake of 5-HT (see BORN, 1970), with subsequent inactivation by MAO that is found in the platelets, appears to have no parallel in smooth muscle. Thus, the sensitivity for 5-HT in the isolated rat stomach preparation is little affected by MAO inhibitors (VANE, 1959), whereas these inhibitors potentiate the preparation to tryptamine and other congeners of 5-HT in which the phenolic hydroxyl group in position 5 on the indole nucleus is absent. It would be interesting to know if these differences find their counterpart in differences in uptake, but to the reviewer's knowledge no such observations have been recorded. Another possible interpretation of VANE'S observations offers itself: possibly the 5-HT is taken up intracellularly (intraneuronally?) by an uptake mechanism resembling the uptake of catecholamines by neuronal vesicles. If tryptamine and similar compounds remain present in the cytosol for a longer period than 5-HT, one would expect them to be exposed to the action of MAO for longer than 5-HT itself. Under these conditions they would be potentiated by an enzyme inhibitor, in contrast to an amine that never reaches a high concentration in the cytosol.

The occurrence of tryptaminergic neurones in the intestinal tract has been discussed as a possibility by several authors (ROBINSON and GERSHON, 1971; ROSS and GERSHON, 1972; FURNESS and COSTA, 1973). However, the only vertebrate species in which the existence of tryptaminergic neurones is well established is the cyclostome, *Lampetra fluviatilis* (BAUMGARTEN et al., 1973).

From what has been said in the preceding part of this section, there still remains much relating to the function of MAO that has to be unravelled. What has been stressed in this section is the relative importance of extra- and intraneuronal inactivation mechanisms. There is no doubt that the intraneuronal disposition of catecholamines (and probably also of 5-HT) is quantitatively of great importance. We have to remember that in the central nervous system the phenomenon of "extraneuronal" enzyme has two counterparts. First, there is the possibility of uptake by glia cells or satellite cells. It is known that these cells contain MAO (see, for instance, SILBERSTEIN et al., 1972), but their contribution to the inactivation of amines is still unknown. Secondly, one could see a counterpart to "extraneuronal" MAO in the presence of enzyme post-synaptically, in the nerve cells, e.g. the ganglion cells in the autonomic ganglia and elsewhere.

Again, the presence of MAO in ganglion cells may be indicative of two different functional aspects. Firstly, enzyme may be required to deal with mediator that has been released preganglionically in the neighbourhood of the cell's receptors, and, secondly, it might be enzyme destined to reach the cell's own axonal endings, by axonal flow.

As to the inactivating action of MAO upon amine set free close to the nerve cells, little can be said about such a function at present. If a cell is "aminoceptive", it might require mechanisms of inactivation, provided there is amine uptake by the effector nerve cell, a possibility of which nothing is as yet known.

The other aspect of intra-ganglionic MAO is the provision of mitochondria to the nerve as a whole and particularly to its endings, by axonal flow. The present ideas on axonal transport have been ably summarized by DAHLSTRÖM (1971). Mitochondria are believed to travel at a rate inferior to that at which small particles, such as vesicles and endoplasmic reticulum, move down the axon, but faster than the rate of transport of soluble proteins (see KARLSSON and SJÖSTRAND, 1971). DAHLSTRÖM (1972) has summarized her findings on MAO in ligated sciatic nerves of rats. There was an accumulation of enzymic activity in the proximal stump which differed in nerves with their sympathetic supply intact and those with the lumbar sympathetic removed. There was an accumulation of MAO activity determined both biochemically using ^{14}C-tyramine as substrate and by the histochemical method of GLENNER et al. (1957). The peak of activity, about 2–2½ times the normal activity, was reached 7 days after ligation in the normal nerve, and 14 days after ligation in the sympathectomized nerve, indicative of a faster rate of flow of enzyme in the adrenergic neurones. There was also some accumulation, biochemically determined, in the distal stump of the nerve. BANKS and MAYOR (1972), using the hypogastric nerve of the guinea-pig and a tetrazolium technique, have demonstrated an accumulation of MAO on both ends of the ligated nerve 16 hrs after ligation.

Ideas on the formation of mitochondria have profoundly changed in recent years. Not so long ago ROBERTSON (1961) was one of the proponents of the view that the mitochondria derived their membranes from the plasma membrane by infoldings of the latter. This view is no longer favoured; mitochondria are believed to arise from other mitochondria, usually close to the nucleus, although the possibility of some new formation of mitochondria in the periphery has not yet been fully excluded (see DAHLSTRÖM, 1972; BARONDES, 1966).

In all this work on axonal transport of MAO two factors have so far not been fully elucidated, although both are mentioned in DAHLSTRÖM's (1972) recent review. *First*, the characteristic location of MAO on the mitochondrion has to be taken into account. It is conceivable that a mitochondrion gets some of its outer cover, believed to be derived from endoplasmic elements, some way down the axon. These elements, we believe, also travel down the axon, but at a rate differing from that of the mitochondria. KREUTZBERG (1969) has recently studied the movement down the axon of the NADH tetrazolium reductase; these observations resemble in their results those on MAO just described.

MCLEAN and BURNSTOCK (1972) have recently studied the flow of MAO, in comparison to that of adrenaline, in the sympathetic fibres of the toad, *Bufo marinus*. Accumulation of adrenaline preceded that of MAO above a ligature, but in both instances the rate was somewhat less than in mammalian nerve. The main interest in their observations is in the fact that a considerable accumulation of MAO was seen, with the technique of GLENNER et al. (1959), at the distal stumps with a maximum of accumulation after four days, whereas the proximal MAO peak developed only after nine days. The cells of the normal 9th ganglion of the sympathetic chain of the toad also gave a strong MAO reaction. The authors discuss the possibility of induction of enzyme but this question cannot be solved until a mitochondrial count in these distal areas has been performed.

It might be mentioned that McLEAN and BURNSTOCK (1972) noted an absence of MAO after ligation in animals in which the sympathetic chain had been removed. This could be used as an argument—and the authors point that out—in favour of a specific association of MAO with adrenergic neurones.

Secondly, in these studies the possibility of characteristic isoenzymes has not been taken into account. Since MAO is present in Schwann cells, some of the enzymic activity tested for in biochemical studies is stationary, and the discovery of substrates attacked at different rates by neuronal and satellite cell enzymes might help in the analysis of enzyme migration along the axon. No mention has as yet been made in this review of the interesting observation on differences in substrate specificity in the developing brain (SHIH and EIDUSON, 1971, 1973); the possibility that some of these isoenzymes may be extraneuronal, e.g. glial, is briefly referred to in a recent review of these observations (EIDUSON, 1972).

As to the functional significance of MAO in invertebrate animals, there was ample evidence of the occurrence of biogenic amines in molluscs that has accumulated *pari passu* with the work on the presence of MAO. Similarly, much was known on the occurrence of amines in annelid worms before MAO was demonstrated in the earthworm (BLASCHKO and HIMMS, 1953). More recently the enzyme has also been found to be present in *Schistosoma mansoni* (NIMMO-SMITH and RAISON, 1968). In echinoderms MAO was found in early experiments (BLASCHKO et al., 1937b; BLASCHKO and HOPE, 1957); it is now well known that biogenic amines occur in this phylum. In other words, there is good general agreement between the occurrence of MAO and amines in invertebrates.

B. Amine Oxidases Related to MAO

The catalysts that can be conveniently discussed under the heading MAO are by no means uniform; this has been fully set out in the preceding pages. We have to recognize that the terminology used may well be superseded in the future, for instance, when we have recognized the functional significance of the "dopamine oxidase" in mammals, the "agmatine oxidase" in *Blaberus*, or the "histamine oxidase" in *Eledone*.

In considering enzymes related to MAO we can say, first of all, that we consider an oxidase as related to MAO when it can be shown to be a flavin-containing amine oxidase. This is a criterion that has the disadvantage that it can only be applied when the enzyme has already undergone a considerable degree of purification. There is, however, one feature in which the substrates of the flavin type of amine oxidases have been found empirically to differ from the non-flavin oxidases to be discussed later in this review. This is the ability of the flavin oxidases to attack secondary amines, an ability never found in enzymes that are not within the flavin group; in fact, in a number of instances this ability has had predictive value.

The few enzymes of this group that have been systematically studied show a wide spectrum of substrate specificity. They illustrate the difficulties of nomenclature of enzymes. The ability to act upon diamines and polyamines not oxidized by MAO is so common in this group that it seems nonsensical to extend the term

"MAO" to cover a group of enzymes which includes many catalysts unable to act upon any monoamine.

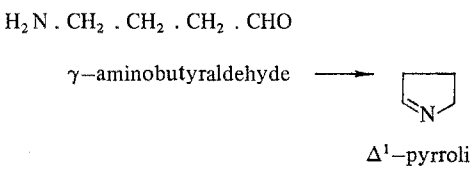
1. Microbial Enzymes Acting on Polyamines

According to TABOR and TABOR (1964) the first report on oxidation of spermine and spermidine by preparations of *Pseudomonas pyocyaneus* (SILVERMAN and EVANS, 1944) was followed by observations made on *Neisseria perflava* (WEAVER and HERBST, 1958) and *Serratia marcescens* (BACHRACH, 1962), on the oxidation of spermidine; the *Neisseria* enzyme also acted on spermine. Both groups of authors noted that in the oxidation of spermidine, propane-1,3-diamine was one of the products of the enzymic reaction. In other words, the enzyme in each organism acted not on one of the terminal amino groups, but severed one of the internal C–N bonds of spermidine. BACHRACH (1962) also established the initial formation of γ-aminobutyraldehyde and formulated the reaction as follows:

$$\underset{\text{Spermidine}}{H_2N \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot NH \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot NH_2} + H_2O + O_2$$

$$\underset{\gamma\text{-Aminobutyraldehyde}}{H_2N \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CHO} + \underset{\text{Propane-1,3-diamine}}{H_2N \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot NH_2} + H_2O_2$$

The aminobutyraldehyde spontaneously cyclized, to give Δ^1-pyrroline, thus:



More recently, TABOR and KELLOGG (1970) have reported more fully on the purification and properties of the *Serratia* oxidase (see also CAMPELLO et al., 1965). TABOR and KELLOGG have found that the *Serratia* oxidase is a particulate enzyme. It contains two cofactors. One of these is flavin adenine dinucleotide (FAD). This group can be readily removed by precipitation in ammonium sulphate at pH 4.5, and the enzyme protein can then be re-activated by adding FAD. The second cofactor is a heme, an iron-protoporphyrin IX. The enzyme has a very interesting substrate specificity, which is illustrated on Table 2.

Of particular interest seems the fine discrimination between the two N-acetyl spermidines: the compound that has the acetyl group attached to the amino group on the three-carbon chain is rapidly oxidized, at about $^2/_3$ of the rate of spermidine, whereas the compound in which the acetyl group sits on the amino nitrogen attached to the four-carbon chain is not oxidized at all. In other words, this enzyme has most exacting specificity requirements, in which the polyamine character of the substrate is of prime importance.

Table 2. Relative rates of oxidation of polyamines by a preparation of *Serratia marcescens*. After TABOR and KELLOGG (1970)

Substrate	Relative rate
Spermidine $NH_2(CH_2)_3NH(CH_2)_4NH_2$	100
Monoacetylspermidine B $CH_3CONH(CH_2)_3NH(CH_2)_4NH_2$	60
N,N′-bis(3-aminopropyl)-1,3-propanediamine $NH_2(CH_2)_3NH(CH_2)_3NH(CH_2)_3NH_2$	48
N-(3-aminopropyl)-1,3-propanediamine $NH_2(CH_2)_3NH(CH_2)_3NH_2$	36
Spermine $NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$	17
Monoacetylspermidine A $NH_2(CH_2)_3NH(CH_2)_4NHCOCH_3$	0

The rate of oxidation of spermidine is taken as 100; the rate with the other amines is in per cent of the rate with spermidine.

Thus, the reaction catalysed by the *Serratia* oxidase severs one of the "internal" C–N bonds of spermidine. Interestingly enough, this is true also for the *Pseudomonas* sp. enzyme studied by PADMANABHAN and KIM (1965), but this enzyme, in contrast to the Serratia enzyme, does not lead to the formation of 1,3-diaminopropane, but to that of 1,4-diaminobutane (putrescine). In other words, these two oxidases cleave the two different bonds between the central nitrogen atom and the two adjoining polymethylene chains. The reaction catalysed by the *Pseudomonas* is:

$$H_2N(CH_2)_4NH(CH_2)_3NH_2 + H_2O + O_2 \rightarrow$$
$$H_2N(CH_2)_4NH_2 + H_2N(CH_2)_2CHO + H_2O_2$$
Putrescine 3-Aminopropionaldehyde

It seems worth mentioning here that two other enzymes active on amines have been described in *Pseudomonas*. The first of these catalyses the oxidative deamination of dimethylamine or related secondary amines in *Pseudomonas aminovorans* (EADY et al., 1971). This is a mixed-function oxidase; it occurs in the organism when grown on media containing methylamine or similar compounds as sole source of carbon. The authors formulate the reaction equation thus:

$$(CH_3)_2NH^+ + NAD(P)H + H^+ + O_2 \rightarrow CH_3 \cdot NH_3^+ + NAD(P)^+ + HCHO + H_2O$$

The enzyme did act on dimethylamine and also on ethylmethylamine and diethylamine, but not on spermidine. The enzyme contains flavin and cytochrome of the P-420 type. In some ways it resembles the mammalian microsomal oxidases, but it is interesting that it contains flavin.

The second enzyme, described by EADY and LARGE (1971) is a dehydrogenase present in *Pseudomonas AM1*, when grown on methylamine as the sole source of carbon. This enzyme, which acts on a number of primary amines, is believed by the authors to contain pyridoxal-5′-phosphate.

2. Microbial Enzymes Acting on Aliphatic Diamines

In 1965 YAMADA et al. (1965d, 1965e) described an enzyme in *Micrococcus rubens* that oxidized putrescine (1,4-diaminobutane). The purification and some of the

properties were more fully described by ADACHI et al. (1966). The *Micrococcus rubens* oxidase acted on putrescine and much more slowly on 1,5-diaminopentane (cadaverine) and on spermidine. Spermine was not a substrate, nor were any of the aliphatic mono-or diamines. The oxidation of spermidine yielded the same products as the *Serratia marcescens* enzyme. The enzyme contained FAD in readily detachable form. More recently, DE SA (1972) has produced a highly purified preparation of the *Micrococcus rubens* enzyme. He reports one mole of flavin per mole of enzyme (M.W. 88000) in his preparation. Addition of substrate under anaerobic conditions led to a loss of the yellow colour of the solution of pure enzyme; so did addition of sodium dithionite. The colour could subsequently be restored by oxygenation. The enzyme was not inhibited by isoniazid, a typical DAO inhibitor, but was inhibited by hydroxylamine and by iproniazid. It was also inhibited by cyanide but not by other metal chelators.

3. Microbial Oxidase Acting on Monoamines

For the student of mammalian MAO a most interesting enzyme is the tyramine oxidase of *Sarcina lutea* described by YAMADA and his colleagues (YAMADA et al., 1967b, c, d; KUMAGAI et al., 1968, 1969a, 1971). These authors have crystallized an enzyme that has a very restricted substrate specificity: of all the many compounds tested only two were oxidized: tyramine and dopamine, at very similar rates. The solution of the pure enzymes looked yellow, and the absorption spectrum was very similar to that of FAD, with two peaks, at 368 and 466 nm. Adding tyramine to the solution of enzyme under anaerobic conditions caused a disappearance of the colour. Heat treatment of the enzyme caused the liberation of a yellow compound that behaved like FAD and replaced FAD as prosthetic group for D-amino acid apo-oxidase. The FAD content was 2 moles per mole of enzyme protein (MW of 129000). No significant metal content was found in the purified enzyme (KUMAGAI et al., 1969a). The enzyme was strongly inhibited by p-chloromercuribenzoate, and also by phenylhydrazine, but not by other carbonyl reagents.

Dopamine, when oxidized by the *Sarcina* enzyme, yielded tetrahydropapaveroline. This condensation reaction between aldehyde and un-oxidized amine was already fully discussed over twenty years ago as a possible way by which an amine oxidase might be involved in the formation of alkaloids in plants (BLASCHKO, 1952a); it has since been studied, using preparations of mammalian MAO, by HOLTZ and his colleagues (for review see HOLTZ and PALM, 1966).

4. Microbial and Mammalian Flavin Enzymes Compared

There are many reasons why these microbial enzymes related to MAO are of great interest to the student of the mammalian enzymes. First of all, there is the interesting variety in substrate specificity of these enzymes. Some of these are acting on monoamines, similar to the mammalian MAO, others on the typical substrates of the mammalian diamine oxidase, and others again on polyamines like spermidine.

It has already been discussed that the *Serratia* enzyme nicely demonstrates the ability to cleave C–N bonds at a secondary amino nitrogen atom. It would be desirable to have information on the chemical nature of the *Pseudomonas* oxidase studied by PADMANABHAN and KIM (1965), which cleaves the other C–N bond at the same nitrogen atom of spermidine. The most important lesson that we can learn from the work on these enzymes is the variety of specificities imposed upon the enzyme, no doubt, by its protein. However, the fact that some of these enzymes accept as substrates secondary amines is a condition presumably imposed on the enzyme by the nature of its prosthetic group.

The prosthetic group, where it is known, is FAD. This is also of interest because it indicates that the formation of the thio-ether link between the iso-alloxazine moiety of the FAD and the cysteinyl residue in the enzyme protein is a development that did not occur in these unicellular organisms.

The occurrence of oxidases similar to MAO has also been reported in protozoa (HUNTER, 1957, 1959a, b, 1961; SHARMA and BOURNE, 1964; JANAKIDEVI et al., 1966; IWATA et al., 1971; IWATA and KARIYA, 1973). These enzymes occur in mitochondria – according to SHARMA and BOURNE (1964) also in some other cell organelles. Their chemistry and function, hitherto entirely unexplored, would seem to represent a most promising field of study.

III. Amine Oxidases Containing Copper and a Carbonyl Group (Pyridoxal)

The heading to this section calls for some comment. The presence of copper has been firmly established in quite a number of these enzymes, in others it is only inferred. The presence of a carbonyl group is a firmly established fact, mainly on the basis of studies of inhibitors of these enzymes. That this carbonyl group is located in pyridoxal, rests on evidence that is fairly satisfactory in some instances but could be improved in others. Pyridoxal-5-phosphate was first discovered as the prosthetic group of the amino-acid decarboxylases, and it is well to remember that even in this family of apparently closely related enzymes there is at least one in which no pyridoxal is present at all but where the carbonyl group is sited in a pyruvyl residue of the enzyme protein. This is the L-histidine decarboxylase of *Lactobacillus 30a* (RILEY and SNELL, 1970). It is therefore advisable, for the time being, to leave the "pyridoxal" in brackets.

Much of the work carried out on one enzyme of this group depended on progress made in the study of another. The enzymes are therefore discussed together. From the chemical point of view, all the copper-containing amine oxidases resemble each other closely in their mode of action, and they share many substrates. On the other hand, their physiological significance is more diverse and much less obvious than that of MAO. A case apart is that of the newly discovered amine oxidase(s) of connective tissue. Here it is the study of enzyme chemistry that is still in its beginnings, whereas the physiological significance is obvious. This

makes it convenient to discuss these connective tissue enzymes separately (in Section V), although they doubtless belong to the same group.

Diamine oxidase (DAO), the histaminase of BEST and MCHENRY (1930), can be considered as the "type specimen" for the whole group. In addition, there are the mammalian plasma oxidases, for which the terms spermine oxidase and benzylamine oxidase have become established. The plasma enzymes have become important for the study of the whole group, as they were the first members of it that were fully purified and crystallized. Furthermore, there are some microbial oxidases, and also the plant amine oxidases about which a considerable amount of information has accumulated. Lastly, there is good reason for the belief that the amine oxidase(s) of both elastic and collagen tissue belong to this group.

In this review the term "histaminase" will be avoided, except where the activity of an enzyme on histamine is referred to. We have already discussed the "histaminase" activity of some preparations of MAO from vertebrate and cephalopod sources. Several enzymes of the group to be discussed here catalyse the oxidative deamination of histamine *in vitro* and we do not as yet know if they function as histaminases also in the intact tissue.

A. Purification and Properties of Copper-Containing Amine Oxidases, in Relation to the Enzyme-Substrate Interaction

Until about 12 years ago, the purification of the enzymes of this group progressed slowly. The first significant success was that of MANN (1961) who achieved a considerable purification of the amine oxidase present in pea seedlings. He was the first to discover that solutions of the highly purified enzyme looked pink, with an absorption peak near 500 nm. The colour disappeared when either putrescine or sodium dithionite were added but reappeared upon oxygenation. Metal chelators inhibited the pea seedling enzyme, and MANN (1961; see also HILL and MANN, 1962, 1964) succeeded, by treatment of the purified enzyme with sodium diethyldithiocarbamate to obtain a protein that was catalytically inactive after removal of the chelating agent but could be reactivated by adding cupric copper.

These discoveries were overshadowed by the successful crystallization of the spermine oxidase of bovine blood plasma (YAMADA and YASUNOBU, 1962a, b), followed by the crystallization of the benzylamine oxidase of pig plasma (BUFFONI and BLASCHKO, 1964). Solutions of both enzymes were pink, with absorption peaks at 480 nm for the bovine and 470 nm for the pig plasma enzymes respectively. Here again the colour was discharged upon adding either benzylamine or sodium dithionite; it reappeared upon oxygenation. Copper was found to be a constituent of both enzymes, and the successful reactivation (by adding cupric copper) of the apoenzyme prepared by dialysis against diethyldithiocarbamate, the method already employed by MANN, was confirmed (YAMADA and YASUNOBU, 1962a). BUFFONI et al. (1968) report a copper content of 2–3 atoms Cu/mole of protein for the pig plasma enzyme, as determined by chemical methods and a content of 1.9–2.2 atoms Cu/mole of protein as determined by electron spin resonance. According to BUFFONI and DELLA CORTE (1972), the crystals of benzylamine oxidase lose copper during storage in ammonium sulphate solution.

The molecular weight of spermine oxidase is about 255000, that of benzylamine oxidase about 195000.

Solutions of the crystalline preparations of the DAO of pig kidney (YAMADA et al., 1967a) had an absorption peak at 470 nm; the pink colour was discharged by putrescine or by sodium dithionite. The molecular weight was determined to be 185000. MONDOVI et al. (1967b) found in a highly purified preparation of the pig kidney enzyme that the copper content was 11–12 mμ moles/mg protein. They determined the molecular weight as between 119500 and 135000 (assuming a partial specific volume of 0.723, as compared with a value of 0.750 used by YAMADA et al. (1967a) in their calculations).

Another of these pink amine oxidases occurs in a fungus, *Aspergillus niger*, when grown on a medium in which amines like n-butylamine represent the sole source of carbon. This enzyme was purified and crystallized by YAMADA et al. (1965a); it had a molecular weight of 252000, and its copper content of 1 g-atom of Cu per 83000 g of protein, was three atoms of Cu per molecule (YAMADA et al., 1965b, c). The pink colour was lost upon adding amine substrate or sodium dithionite and restored on oxygenation. Diethyldithiocarbamate removed the copper; the activity of the residual protein could be restored by adding cupric sulphate.

In summary, it can be said that the copper content and the spectral properties of these oxidases are well establised. The presence of copper is responsible for the fact that copper chelators act as inhibitors of the enzymes.

There is another group of inhibitors for this group of oxidases: carbonyl reagents. The inhibitory action of this group of compounds made ZELLER suspect at an early stage that the active centre of DAO contained a carbonyl group. Later on WERLE and VAN PECHMANN (1949) suggested that this group was located in pyridoxal phosphate. The older references are fully reviewed by KAPELLER-ADLER (1970).

The pioneer work by GORYACHENKOVA and ERSHOVA (quoted from GORYACHENKOVA et al., 1968) on the vitamin B_6 activity of hydrolysates of fairly highly purified preparations of the DAO of pig kidney, in which only traces of activity were found, was followed by observations on the crystals of pig plasma benzylamine oxidase (BLASCHKO and BUFFONI, 1965) in which hydrolysis by acid yielded low-molecular-weight products that activated the L-tyrosine apodecarboxylase of *Streptococcus faecalis R;* a higher degree of reactivation was obtained when ATP was added to the incubates and the bacterial preparation was not washed to remove the enzyme required for phosphorylation of pyridoxal. These observations indicated that in the hydrolysis procedure some of the pyridoxal-5-phosphate was hydrolysed.

Similar experiments were carried out by MONDOVI et al. (1967a) who hydrolysed their highly purified pig kidney DAO preparation and found that a product obtained in presence of both diethyldithiocarbamate and phenylhydrazine activated the apoenzyme of aspartate aminotransferase. They calculated that the pyridoxal phosphate content of the enzyme was one mole of pyridoxal phosphate for one atom of Cu, or one mole per unit of 90000 molecular weight.

The spectroscopic evidence has led other observers to suggest that the prosthetic group of the pink amine oxidases contains pyridoxal-5-phosphate. However,

WATANABE et al. (1972) do not think that the identity of the chromophore group with pyridoxal-5-phosphate is fully established. BUFFONI and DELLA CORTE (1972) have proposed that pyridoxal is held in covalent linkage to the enzyme protein; such a link may be responsible for the great difficulty in detaching a low-molecular-weight component.

A novel method was initiated by BUFFONI (1968) in the study of pig plasma benzylamine oxidase, and this method was again used by KUMAGAI et al. (1969b) for pig kidney DAO. Both groups incubated solutions of crystalline enzyme with ^{14}C-labelled histamine under anaerobic conditions. The incubate was, still in the absence of oxygen, reduced with sodium borohydride. The protein that was then removed from the incubation mixture was radioactive. It was hydrolysed, and the radioactive hydrolysis product was compared by electrophoresis with synthetic ^{14}C-labelled pyridoxylhistamine; the hydrolysis product and the synthetic compound migrated to the same position on electrophoresis.

As to the mechanism of the enzyme reaction, WERLE and VON PECHMANN (1949) suggested that the interaction between oxidase and amine substrate involved the formation of an imino bond between the amino groups of the substrate and the carbonyl group of pyridoxal-5-phosphate. This was followed by a cleavage, leaving aldehyde and the pyridoxal groups as pyridoxamine phosphate. This internal transamination was followed by an oxidative deamination of the pyridoxamine form of the enzyme to the pyridoxal form. The original suggestion, that a flavin moiety was responsible for the oxidative step, has not been borne out in subsequent work, since no flavin has been found in the fully purified oxidases of this group. However, the role of the copper in the reaction is still under active discussion (HAMILTON, 1968; MONDOVI et al., 1969; TAYLOR et al., 1972; BUFFONI et al., 1972).

WERLE and VON PECHMANN'S suggestion of the initial reaction mechanism of the copper-containing oxidases is supported by observations on the *Aspergillus* oxidase (YAMADA et al., 1972). Titration of the enzyme with phenylhydrazine suggests that there are two carbonyl groups per mole of enzyme; this conclusion is based on the appearance of a sharp band with a peak at 442.5 nm when phenylhydrazine is added, with a change of the colour from pink to yellow (a similar peak has been reported for the other copper amine oxidases studied). Equally, when substrate (n-butylamine) was added under anaerobic conditions, full bleaching of the *Aspergillus* enzyme was seen when two moles of amine per mole of enzyme were added. Neither ammonia nor hydrogen peroxide were formed in the absence of oxygen.

Since the pig kidney enzyme does not contain flavin, copper has been implicated in the oxidative deamination of the pyridoxamine phosphate formed in the first stage of the enzymic reaction (see MONDOVI, 1972).

It should be emphasized that there are some differences in the reports from different observers on the amounts of copper and pyridoxal phosphate present. LINDSTRÖM and PETTERSSON (1973) have recently titrated the carbonyl groups in pig plasma benzylamine oxidase, using phenylhydrazine. Under these conditions they confirm earlier observations on the spectral changes that occur, but they report that these changes were completed when 1 mole of phenylhydrazine had reacted with one mole of enzyme. On the other hand, the copper content of the

enzyme was determined as 2.5–2.7 moles of Cu per mole of protein. This is at variance with the ratio of 1:1 for copper and pyridoxal reported by MONDOVI et al. (1967a) with DAO of pig kidney.

MONDOVI et al. (1969) have reported that some reduction of the copper in DAO occurs upon the addition of substrate; these changes are reversed upon oxygenation.

For the role of copper see also BUFFONI (1968); LINDSTRÖM, OLSSON and PETTERSSON (1973, 1974); FINAZZI-AGRO et al. (1969); COSTA et al. (1971); YAMASAKI et al. (1970).

B. Substrate Specificity of Amine Oxidases Containing Copper and a Carbonyl Group

We distinguish in this group of enzymes specificity requirements imposed by the nature of the prosthetic group from those accounted for by the chemical properties of the enzyme proteins.

One characteristic property distinguishes all the oxidases of the copper group from the flavin oxidases: the copper enzymes will accept only primary amines as substrates. Early work on MAO centred around the ability of the enzyme to act upon adrenaline, a secondary amine (BLASCHKO et al., 1937a, b). The enzymes of the copper group which act upon histamine do not oxidize ω-N-methyl histamine (KAPELLER-ADLER and IGGO, 1957; BUFFONI, 1966). In contrast, we have already discussed that the MAO present in the renal appendages of *Eledone cirrhosa*, which oxidizes histamine, acts also upon ω-N-methyl histamine (BOADLE, 1969). Inhibitor studies show that the *Eledone* enzyme is of the flavin type. We see in the restricted substrate specificity of the copper enzymes a requirement accounted for by the nature of the prosthetic group: the interaction between substrate and enzyme involves the formation of a bond between the amino group of the substrate and the carbonyl group of the enzyme, situated presumably in pyridoxal-5-phosphate (BLASCHKO and BOADLE, 1968). The interaction results in the formation of a Schiff's base, thus:

$$R \cdot CHO + H_2N \cdot CH_2 \cdot R^1 \rightarrow R \cdot CH{:}N \cdot CH_2R^1$$

where R stands for pyridoxal phosphate and R^1 for part of the substrate.

If we trace what is known of the amines that are attacked by enzymes belonging to the group of copper-containing catalysts, we see that this general rule is obeyed. The contrast between flavin enzymes and copper-pyridoxal oxidases becomes particularly clear when the oxidation of polyamines like spermine or spermidine is considered. The flavin group contains enzymes, already discussed, that act on these compounds: they cleave internal C–N bonds in the polyamine molecule. In other words, they attack the substrate at a secondary amino group. On the other hand, the copper enzyme active on spermine and spermidine, the spermine oxidase of bovine plasma, oxidizes the polyamines at one of the terminal, primary, amino groups. This was not known in 1962, when the mammalian plasma oxidases were first reviewed (BLASCHKO, 1962). An *ad hoc* hypothesis was proposed to account

for the action of spermine oxidase on spermine, a reaction in which spermidine was then believed to be one of the primary reaction products. This hypothesis became unnecessary when TABOR et al. (1964) repeated earlier experiments, but using more highly purified enzyme preparations, and demonstrated that spermidine was not the first product of the oxidative deamination of spermine by the bovine plasma enzyme. With both spermine and spermidine as substrates the primary oxidation products were the terminal aldehydes; these unstable compounds were characterized after reduction with sodium borohydride to the corresponding alcohols. The product of oxidation of spermine was a dialdehyde, that of spermidine a monoaldehyde. The reactions equation were formulated as follows:

$$\underset{\text{Spermine}}{NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2} + 2O_2 + 2H_2O \rightarrow$$

$$HC(:O) \cdot (CH_2)_2NH(CH_2)_4NH(CH_2)_2CHO + 2NH_3 + 2H_2O_2$$

$$\underset{\text{Spermidine}}{NH_2(CH_2)_3NH(CH_2)_4NH_2} + O_2 + H_2O \rightarrow$$

$$HC(:O) \cdot (CH_2)_2NH(CH_2)_4NH_2 + NH_3 + H_2O_2$$

Thus, the bovine spermine oxidase obeys the general rule; it acts exclusively on terminal, primary, amino groups.

The inability to act on secondary amines is shared by all oxidases of this group. For example, the crystalline preparation of amine oxidase of *Aspergillus niger* will oxidize noradrenaline, but it is without action on adrenaline (YAMADA et al., 1965b). Similarly, it is known that the plasma oxidases, both spermine and benzylamine oxidase, will not act on the N-methylated derivatives of benzylamine, β-phenylethylamine and γ-phenylpropylamine, whereas the primary amines are readily attacked (BLASCHKO, 1960). Also the amine oxidase of pea seedlings acts upon benzylamine and β-phenylethylamine, but not on N-methylbenzylamine or β-phenylethylmethylamine (HILL and MANN, 1964). The plant enzyme also acts upon noradrenaline but not on adrenaline (WERLE et al., 1961).

It should be mentioned here that there exists one report on an oxidase that seems to be not in agreement with the general rule: ADACHI and YAMADA (1970) have studied the amine oxidase of a fungus, *Fusarium culmorum*. The mycelia of this fungus, grown on a medium containing n-butylamine, yielded an enzyme that was purified about 6000 times. The enzymes was inhibited by copper chelators and by carbonyl reagents; however, it was also strongly inhibited by iproniazid, a typical MAO inhibitor. This preparation oxidized methylbenzylamine more rapidly than benzylamine. More observations on this enzyme must be made until its classification can be considered as secure.

The specificity rules that rest on the interaction between prosthetic groups and substrates are common to all enzymes of this group. On the other hand in their substrate specificity, inasfar as the latter is determined by the interaction between substrate and enzyme protein, the copper-containing oxidases display a variety as wide as that found among the flavin oxidases.

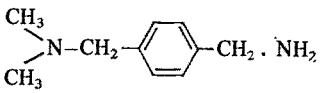
The mammalian DAO is a much studied member of this group. It was first described as histaminase, and very probably histamine is an important substrate

of the enzyme *in vivo*. The pig kidney has served as a source of enzyme in many studies, but there is also work on the placenta enzyme, and recently studies have been made of the histamine of rabbit liver (ARGENTO-CERU et al., 1973a, b).

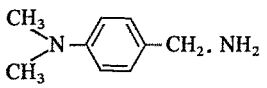
Observations on the relative rates of oxidation of histamine and cadaverine at different values of pH led to the conclusion that it was the di-cationic form of histamine that was accepted as the substrate of the pig kidney enzyme. This suggestion has recently received support in observations carried out on more highly purified preparations of the enzyme (BARDSLEY et al., 1970).

It is not intended here to give a full review of the work on the substrate specificity of DAO. ZELLER'S contribution to this topic has been repeatedly reviewed (for references see ZELLER, 1972). A full discussion of DAO is found in BUFFONI'S (1966) review.

The main outcome of this work is that the mode of attachment of the two amino groups to the enzyme is different. One amino group attaches itself to the prosthetic group; this is the primary amino group that is recovered in the oxidation reaction. The other amino group is attached, presumably by ionic bonds, to the enzyme protein. This second group needs not to be a primary amino group. An important substrate in which the second basic group is not an amino group, is histamine. Another interesting example has recently been given by BARDSLEY et al. (1972), who have shown that p-dimethylaminomethylbenzylamine is rapidly oxidized by DAO.



On the other hand, p-dimethylaminobenzylamine is not significantly attacked (BARDSLEY et al., 1973).



In this latter compound the dimethyl amino group directly attached to the aromatic ring is presumably not protonated at the pH used in these studies. Incidentally, the work of BARDSLEY and his colleagues has corrected some earlier observations according to which p-xylylenediamine was not a substrate of the pig kidney oxidase (BLASCHKO and CHRUSCIEL, 1959).

DAO takes its name from the fact that it acts upon short-chain aliphatic diamines like putrescine and cadaverine. On the other hand, the monoamines are either not oxidized at all or only at extremely high concentrations. The pea seedling oxidase shares with DAO the ability to act on these diamines, but its action on the aliphatic monoamines is not negligible. Also, the pea seedling oxidase acts upon the polyamines, spermine and spermidine, substances not significantly acted upon by the pig kidney oxidase. Another plant oxidase of the same group, that of barley and maize seedlings, acts exclusively upon spermine, less on spermidine or 3,3′-diaminodipropylamine, but not on the aliphatic amines (SMITH, 1970).

At the other end of the range we find the amine oxidase of *Aspergillus niger* (YAMADA et al., 1965b); this is an adaptive enzyme found in mycelia grown in the prescence of n-butylamine. The oxidase acts rapidly only on aliphatic monoamines, very slowly on diamines and not at all on spermine or spermidine. (In a preliminary publication YAMADA et al., 1965e noted a somewhat stronger activity on the short-chain diamines when the oxidase was prepared from mycelia grown in the presence of putrescine as source of carbon, but this interesting observation has not been followed up any further.)

The amine oxidases of mammalian plasma exhibit characteristic substrate specificities. The specificity pattern differs from species to species, but some general properties are obvious. There is, first of all, the difference between spermine oxidase and benzylamine oxidase. It has already been discussed (BLASCHKO, 1962; BLASCHKO and BONNEY, 1962) that the spermine oxidase is restricted to the ruminants and tylopods, and a few other animals, viz. the hippopotamus and the hyracoidea. The biochemical relationship between the hippopotamus and the ruminants has recently been strengthened by the finding that the hippopotamus differs from the pig in the presence of arginine-vasopressin in the posterior lobe of its pituitary gland (UTTENTHAL and HOPE, 1972).

As to the amine oxidase activity of the plasma enzymes they share with MAO the ability to act on aliphatic monoamines and the long-chain diamines; the short-chain diamines are either not acted upon at all or only very poorly. Apart from these general properties, there are many minor species differences. The crystallization of the pig plasma enzyme was embarked upon because in this species the oxidase rapidly attacks two amines of great pharmacological interest: mescaline and histamine. Among the animals that contain spermine oxidase, the llama is characterized by the ability to act on mescaline. In some other species mescaline and histamine are not oxidized at a significant rate.

The lesson to be learnt from the substrate specificity of the enzymes of the copper-pyridoxal group reinforces what emerged from the study of the flavin enzymes: substrate specificity, inasfar as it is based upon the interaction between substrate and enzyme protein, varies from enzyme to enzyme. The protein of the diamine oxidases has probably a negatively charged group that interacts with the second basic group in the substrate molecule; this second basic group may be another primary amino group, but it can also be substituted, as in a methylamino or dimethylamino group, or it may be located in an imidazole ring. On the other hand, the monoamine oxidases among these enzymes lack such an anionic group; here the enzyme protein presumably interacts with the monoamine substrate in an entirely hydrophobic region. It has been repeatedly emphasized that such a hydrophobic region is also present in DAO, interposed between the pyridoxal group and the anionic group; this has already been referred to.

A recent observation on benzylamine oxidase should be mentioned: TAYLOR et al. (1972) have found that the benzylamine oxidase of pig plasma also acts upon benzyl alcohol. It remains to be seen if this ability to act upon alcohols is a general property of this class of oxidases. The ability of the enzyme to accept an alcohol as substrate is reminiscent of the slow action of D-amino-acid oxidase on D-α-hydroxy acids (for references see YAGI et al., 1969).

C. Functional Significance of Copper-Containing Amine Oxidases

DAO was first described as histaminase. We have seen that histaminase activity is a property of several enzymes, and experiments already referred to (BERTACCINI, 1961; BOADLE, 1969), show that in molluscs a flavin-containing histaminase is at work *in vivo*.

The question as to the significance of DAO is closely bound up with the problem, as yet unresolved, of the physiological rôle of histamine. A strong case in favour of a role of histamine, particularly in tissue growth and repair, has been presented by KAHLSON and ROSENGREN (1971). This is based chiefly on the study of the "histamine-forming capacity" of animal tissues. This attractive case still awaits definitive confirmation. Since the publication of KAHLSON and ROSENGREN'S monograph there have been observations on the histamine-H_2 receptors. The finding by BLACK et al. (1972) of an inhibition of gastric secretion, both pentagastrin-induced and food-induced, by burimamide strongly supports the thesis that histamine has a physiological rôle in gastric secretion.

DAO is a factor that determines the magnitude of the response of excitable tissues to histamine. This was shown by ARUNLAKSHANA et al. (1954), who described the potentiation of the effect of histamine by DAO inhibitors, in isolated organs as well as upon the cat's blood pressure. The inhibitors studied included several carbonyl reagents, such as semicarbazide or aminoguanidine, but also cadaverine, a substrate of DAO that interferes competitively with the inactivation of histamine. On the guinea-pig's ileum, the response to histamine was found to be potentiated by the picolylamines (BLASCHKO and KURZEPA, 1962), substances that were poor substrates, but moderately strong inhibitors, of DAO. On the other hand, the N-methylated derivative of 4-picolylamine had a low affinity for the enzyme, and it did not show the characteristic potentiating effect of the primary amines. Thus, DAO is a limiting factor in the response to histamine.

The full significance of DAO in vertebrates is far from clear. It might be mentioned here that high DAO activity has been demonstrated in the brains of several teleost fishes (BURKARD et al., 1963); in the mammalian brain such activity appears to be absent.

Oxidative deamination is not the only reaction in which histamine loses its biological activity. Two other types of reaction have been described: N-acetylation and N-methylation. All these reactions have been discussed by KAHLSON and ROSENGREN (1971). The primary product of the methylation reaction is 1,4-methylhistamine. This compound is further metabolised, by amine oxidases, MAO as well as DAO (LINDELL and WESTLING, 1957), and it is also a substrate of the two plasma oxidases, spermine oxidase and benzylamine oxidase (BLASCHKO, 1959). In the intact organism, MAO seems to be the main catalyst that metabolizes 1,4-methylhistamine (see SCHAYER, 1959; ELIASSEN, 1973).

Whereas 1,4-methylhistamine is pharmacologically relatively inert as a histamine-like agent, the ω-N-methyl derivative has long been known to be highly active. It is important to note that this compound, as a secondary amine, may be more active because it is not a substrate of the copper-containing amine oxidases. On the other hand, it is a substrate of the molluscan histaminase which is a flavin enzyme (BOADLE, 1969).

The part played by the plasma oxidases in the biological inactivation of histamine is still uncertain. In some species, e.g. the pig, the histaminase activity is readily demonstrated, in others, e.g. in man (MCEWEN, 1972), it is not very prominent. It is possible, therefore, that the contribution of the plasma oxidases to the biological inactivation of histamine depends on the species. Also, there are species where different individuals have very different enzymic activities. For instance, in dog plasma one finds great individual variations in the rate of oxidation of histamine (BLASCHKO, 1959).

KAHLSON and ROSENGREN (1971) have fully discussed the conditions in which histaminase activity appears in the blood and tissue fluids. The earliest report is that of the appearance of histaminase activity in pregnant women. It seems likely that the placenta is the source of this enzyme. (For a recent study of the human placenta oxidase see PAOLUCCI et al., 1971.)

Another appearance of a soluble histaminase was reported from KAHLSON'S laboratory over 20 years ago. CARLSTEN (1950) described the release of histaminase into the thoracic duct lymph of the cat after adrenalectomy. This release of enzyme was inhibited by cortisone. More recently, SCHMUTZLER et al. (1966b) have studied the release of a histaminase during anaphylactic shock in the guinea-pig (see also CODE et al., 1961). SCHMUTZLER et al. (1966b) have shown that the liver is the source of this enzyme. The release appears to be mediated by heparin, a substance that has a powerful releasing effect on the histaminase, not only in the guinea-pig (SCHMUTZLER et al., 1966a) but also in many other vertebrate species (HANSSON and THYSELL, 1968). The enzyme active under these conditions has diamine oxidase activity; it acts upon putrescine (see also DAHLBÄCK et al., 1968), but in a study of the portal vein blood of the guinea-pig (SCHMUTZLER and KNOP, 1968) not only histamine and diamines were oxidized but also some monoamines, and some polyamines. The possibility can therefore not be excluded that a number of soluble oxidases, normally intracellular, are mobilized by heparin. This is a possibility that has gained fresh interest in view of the work on connective tissue oxidases to be discussed later in Section V.

The diamine and polyamine oxidase activities of many mammalian oxidases of the copper group were until recently of little interest. This situation has changed in recent years, because more has been learnt of the occurrence of the substrates of these enzymes in animal tissue.

Polyamine biosynthesis was first studied in microorganisms (see BACHRACH, 1970) but it is now known that similar reactions occur in animal tissues (RAINA, 1963). The amine precursor of the polyamines is 1,4-diaminobutane (putrescine). This compound is transformed to spermidine by the addition of one $-C-C-C-N$ moiety, and to spermine by the addition of a second such group. Putrescine itself is the product of the reaction catalysed by the enzyme L-ornithine decarboxylase (PEGG and WILLIAMS-ASHMAN, 1968a). Thus, putrescine, an excellent substrate of DAO, is a normal metabolite in the animal body. Moreover, putrescine stimulates the activity of S-adenosylmethionine decarboxylase, the enzyme responsible for providing the propylamino residue required for spermidine synthesis from putrescine (PEGG and WILLIAMS-ASHMAN, 1968b; see also HANNONEN et al., 1972b).

Since putrescine arises as an intermediate of polyamine biosynthesis, the activity of the amine oxidases on these two polyamines deserves a more detailed scrutiny than it has received in the past. When this field was last reviewed (BLASCH-

KO, 1962) it was pointed out that it was probable that the source of polyamine was within the animal body; this has now been amply confirmed (RAINA, 1963; HANNONEN et al., 1972a). As to the degradation of spermine and spermidine, nothing essentially new has been added to the observations then discussed. It seems likely that some degradation of the two polyamines occurs also in the tissues of those animals in which no plasma spermine oxidase is present (see RAINA, 1963), but the nature of catalyst involved is unknown. As to the physiological function of spermine oxidase, the proposition was put forward that the very high activity of the plasma oxidase against the polyamines was evolved in those animals in which a large amount of polyamine was released from the decaying micro-organisms of the gastro-intestinal tract. A typical spermine oxidase activity has only been observed in those animals in which special and characteristic compartments for the fermentation of cellulose have been evolved. These animals include all ruminants and tylopods, the hippopotamus and the hyracoidea. No new observations to support or refute this idea have been adduced since this problem was discussed (BLASCHKO, 1962).

It might be recorded here that the mucosa of the rumen also contains MAO (SCHULZ, 1963).

The functional significance of the plasma amine oxidases must still be considered as obscure. It seems worthwhile pointing out that dopamine is an excellent substrate of these enzymes (DELLA CORTE and PERRINO, 1968; MCEWEN, 1965; 1972). One of the interesting properties of the plasma oxidases is that in many species they make their appearance rather late during postnatal development (BLASCHKO and HAWES, 1959; BLASCHKO and BONNEY, 1962). This property is of interest in relation to the connective tissue oxidase(s), to be discussed below: if the plasma enzymes are a product of the degradation or secretion of the cells of the connective tissue, one could understand that they are not released when the formation of new collagen and elastic fibres is at its maximum.

It was the relationship between copper and plasma amine oxidase activity which led to the study of benzylamine oxidase activity in copper-deficient pigs. It was found that in the deficient animals the enzymic activity was absent or almost absent (BLASCHKO et al., 1965). When copper was added to the diet, activity appeared in the plasma.

A full discussion of the significance of the copper-containing oxidases in plants is outside the scope of this review. Since the publication of the comprehensive article by SMITH (1971) there has been a report on the occurrence of *sym*-homospermidine, $H_2N(CH_2)_4NH \cdot (CH_2)_4NH_2$, in sandal (*Santalum album* L.) (KUTTAN and RADHAKRISHNAN, 1972). SMITH (1971) discusses some of the ideas on the participation of amine oxidase in alkaloid biosynthesis. Over twenty years ago, the author pointed out that the occurrence, side by side, of amine and aldehyde might be of relevance in the formation of tetrahydropapaverine (BLASCHKO, 1952a). The possibility that tetrahydropapaveroline or related compounds may occur in animal tissues, especially after medication with massive doses of L-dopa, is still under discussion (see HOLTZ et al., 1964; HOLTZ and PALM, 1966; SANDLER, 1972).

The rise of amine oxidase activity in germinating plants has been noted for a long time (see SMITH, 1971). It is of interest that this rise is paralleled by a rise in histamine-forming capacity (KAHLSON and ROSENGREN, 1971).

IV. Transformation

During the past decade, a number of papers from GORKIN's laboratory have appeared in which the transformation of amine oxidases has been described (for references see GORKIN, 1972). These experiments have been carried out on preparations of enzymes of both groups, those containing flavin as well as those containing copper and – presumably – pyridoxal phosphate. Two flavin-containing oxidases were studied: mitochondrial MAO and the tyramine oxidase of *Sarcina lutea*. Treatment with oxidizing agents reduced the ability of the preparations to act upon tyramine. The beef liver mitochondrial preparation acquired upon treatment with oxidizing agents an ability to act upon cadaverine, histamine, spermidine and lysine. Similarly, the *Sarcina lutea* preparation acquired the ability to act on lysine, putrescine and spermine.

Also, preparations of pig kidney DAO were treated with reducing agents. Under these conditions their ability to act on putrescine was reduced whereas they acquired the ability to act on tyramine.

GORKIN has suggested that these phenomena are related to an interaction of the "transforming" agents with sulphydryl groups: oxidizing agents convert-SH groups present into-S–S-bridges; reducing agents re-establish-SH groups originally present. Conversely, the reducing agents increase the number of -SH groups present in the pig kidney oxidase; this increase is counteracted by treatment with *o*-iodosobenzoate.

A confirmation of GORKIN's earlier observation on the shift of the relative rates of oxidation of nitrophenylethylamine derivatives by mitochondrial MAO under the influence of oxidizing and reducing agents has recently been reported by ZELLER (1973).

A satisfactory interpretation of these observations will presumably have to await a full understanding of the structure of the amine oxidases. However, in the light of what has been learnt from the study of the specificity of the enzymes involved, we can say that it appears that the reagents used may cover or uncover groups in the active centres of the enzymes studied. For instance, the idea proposed by ZELLER and discussed above, is that DAO contains an anionic group that interacts with the second basic group present in the diamine substrate. Possibly, when access to this group is prevented, the relative rate of oxidation is shifted in favour of monoamines, and their attachment to the enzyme protein may even be facilitated. Conversely, one might have to postulate that the treatment of the flavin-containing enzymes with oxidizing agents unmasks in the mitochondrial MAO and in the *Sarcina* oxidase a charged group able to interact with the second basic group present in the diamines.

It is more difficult to account for some of the other observations described. For instance, STESINA et al. (1971) have reported that a purified preparation of pig kidney DAO, treated with H_2S, had not only a decreased ability to oxidize diamines or histamine but had also acquired the ability to act upon N-methyl-β-phenylethylamine. Since it is believed that the inability of DAO to act on the secondary amines is due to the properties of the carbonyl group in the active centre, one would have to postulate that the enzyme has changed its cofactor. The use of fully purified enzyme would exclude such a possibility.

According to GORKIN, the treatment of MAO preparations also alters inhibitor specificity. These observations are of interest in relation to earlier reports, according to which the MAO of frog's liver acquires sensitivity to semicarbazide upon exposure to oxygen, especially at 37° C (SMITH, 1960). In discussing his observations, SMITH concluded that semicarbazide inhibition was "dependent upon the prior oxidation of some sulphydryl groups of the enzyme". In his experiments the semicarbazide inhibition was non-competitive.

It might be mentioned in this context that BOADLE (1967) has also found some inhibition by semicarbazide of the monoamine oxidase of the liver from *Petromyzon marinus* and of some elasmobranch and teleost fishes. In *P. marinus* liver the percentage inhibition differed according to the substrate tested. These preliminary observation merit further examination in the light of the knowledge on isoenzymes that has since accumulated.

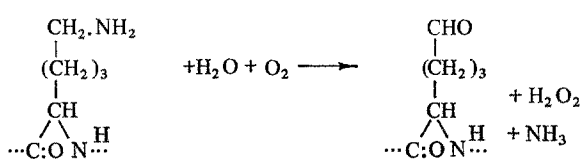
V. The Role of Amine Oxidases in Connective Tissue

Whereas the study of the oxidases concerned with the biological inactivation of the biogenic amines goes back over a span of forty years and more, knowledge of the existence of the connective tissue oxidase is of very recent origin. As yet, very little is known of the enzymes themselves; much of our knowledge comes from a study of the chemical composition of elastin and collagen, and of the factors that interfere with the formation of the mature proteins.

A. The Chemistry of the Cross-Links in Connective Tissue

The two connective tissue proteins are characterized by the presence of cross-links, absent in the immature proteins, that connect the individual peptide chains. These cross-links are essential for the mechanical properties of the proteins in the extracellular space (see PIEZ, 1968).

Although the study of the cross-links is by no means complete, it turns out that all known links depends for their formation upon the activity of an enzyme that catalyses the oxidative deamination of a lysyl residue in the peptide chain, with the removal of the ε-amino group. In this reaction the lysyl residue is converted to an aldehyde. For this residue the term "allysyl" is now in general use (PINNELL and MARTIN, 1968). The reaction can be formulated thus:



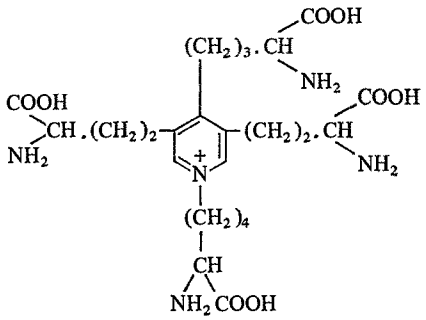
Lysyl Lysyl Oxidase Allysyl

In collagen, some of the lysyl residues in the α-chains of tropocollagen are first converted to hydroxylysyl; these can also yield "hydroxyallysyl" residues. The conversion of the lysyl (or hydroxylysyl) residues to the corresponding aldehydes is catalysed by enzymes for which the term "lysyl oxidase" has been introduced (MARTIN et al., 1970). This term refers to one important feature of the enzyme(s), i.e., to the fact that the natural substrate is a lysyl (or hydroxylysyl) residue that is built into a peptide chain.

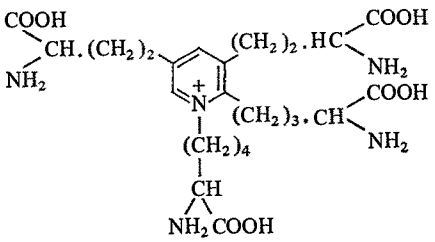
Elastin and tropocollagen have very different mechanical properties, and although the initial enzymic reactions essential to the formation of a cross-link are similar, the cross-links in the two types of tissue are different. In particular, in elastin more of the cross-links are irreversible. This is due to the fact that the initial reaction, in which the bridge is formed, is followed by a second reaction in which the link is rendered irreversible. Collagen, on the other hand, is a compound that undergoes catabolic changes readily, and here many of the links are reversible.

1. Cross-Links in Elastin

The first evidence on the chemical nature of the cross-links in elastin came when two new amino-acids were discovered in hydrolysates of elastin to which the names desmosine and isodesmosine were given (PARTRIDGE et al., 1964; 1965). Both compounds contain a pyridinium ring, with four side chains, each of which ends in an α-amino acid residue. PARTRIDGE (1965) clearly recognised that these compounds could be formed from the condensation of one lysyl residue with three allysyl residues. The initial condensation product, it is believed, is stabilized by



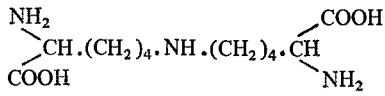
Desmosine



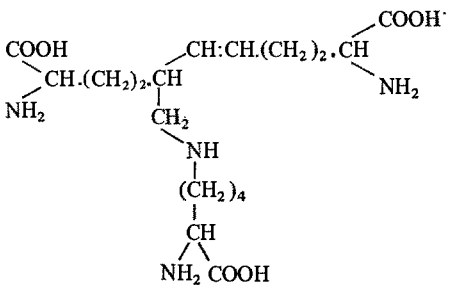
Isodesmosine

reduction of an unsaturated intermediate, to form an aromatic ring. This interpretation is supported by the discovery of merodesmosine, a compound that arises from the fusion of a lysyl residue with two allysyls (STARCHER et al., 1967).

It is easy to see that the desmosines can cross-link as many as four different peptide chains. A simpler cross-link was discovered in hydrolysates of bovine ligamentum nuchae; this is lysinonorleucine (FRANZBLAU et al., 1965). Lysinonorleucine arises from the interaction between one lysyl and one allysyl, with the resulting Schiff's base subsequently reduced. This interpretation is supported by the finding that upon the reduction of elastin by sodium borohydride the yield of lysinonorleucine was increased, indicating that the unreduced Schiff's base was also present (LENT and FRANZBLAU, 1967).



Lysinonorleucine



Merodesmosine

More recently FRANZBLAU et al. (1970) have given evidence of the occurrence of a third type of cross-link in elastin. According to their findings this quantitatively perhaps most important cross-link is an aldol, a condensation product arising from the interaction of two allysyl residues:

$$COOH \cdot CH(NH_2) \cdot (CH_2)_2 \cdot C(CHO):CH \cdot (CH_2)_3 \cdot CH(NH_2) \cdot COOH$$

This compound may not only serve as cross-link, but also as a precursor of the merodesmosine molecule.

All the evidence here related is fairly recent, and it seems probable that more cross-links will be found in the future. There has been a very recent report on a link in which, in addition to the allysyls, a histidyl residue is also involved (TANZER et al., 1973).

2. Cross-Links in Collagen

More is known about the chemistry of collagen, but less of that of the cross-links. The occurrence of amino-aldehyde in collagen was demonstrated before the nature

of the cross-link was demonstrated (see GALLOP et al., 1968, 1970). Collagen is a macromolecular assembly of tropocollagen molecules, each of which consists of a triple helix of three peptide chains. These tropocollagen molecules are diagonally arranged in a staggered fashion. There usually are two α-chains, α_1 and α_2, and one β-chain. For the α-chains it has been established that each has an N-terminal and a C-terminal end, each non-helical. It is these non-helical regions of the chains that seem to be particularly important in cross-linking.

Two kinds of cross-links are distinguished in collagen: *inter*molecular and *intra*molecular links. The latter are bonds between the individual chains in one tropocollagen, the former bridges between different tropocollagen molecules.

As in elastin, all the cross-links that have been identified are formed subsequent to a reaction in which an ε-amino group of one or more lysyl or hydroxylysyl residues are oxidatively removed. As in elastin, subsequent to the formation of the allysyl or hydroxyallysyl residue, the link formed is an aldimino or an aldol bond.

As to the intramolecular bonds, that which is best characterized is situated in the non-helical N-terminal portion of the α-chains. This bond is one between two allysyl residues located in position 9 on the α_1-chains (see BORNSTEIN and PIEZ, 1966; PIEZ et al., 1970). The two residues form an aldol compound that acts as a bridge between identical position on two peptide chains of the tropocollagen molecule.

For the cohesion of the collagen fibres the *inter*molecular bonds seem to be of particular importance. BAILEY et al. (1970a) distinguish two types of these bonds: first, there is a reversible cross-link, present in soluble collagen. Its reduction renders the collagen insoluble. The compound responsible for this link was, after reduction, isolated and identified as N^{ε}-(5-amino-5-carboxypentyl) hydroxylysine

$$COOH \cdot CH(NH_2) \cdot (CH_2)_2CHOH \cdot CH_2 \cdot NH(CH_2)_4CH(NH_2) \cdot COOH$$

In other words, one lysyl and one hydroxylsyl are involved, one of them after deamination by an amine oxidase (BAILEY and PEACH, 1971). In contrast to what happens in elastin tissue, the Schiff's base is not reduced in collagen.

In addition, a second intermolecular bond has been described in collagen, which appears to be more stable (BAILEY et al., 1970b); this compound, which has been called syndesine, is believed to arise from an aldol formed between the δ-semialdehydes derived from one lysine and one hydroxylysine. This bond seems to be most readily demonstrated in less soluble types of collagen.

There are some indications that an increasing degree of hydroxylation of the lysyl residue renders the collagen more soluble (BARNES et al., 1971).

As to the location of lysyl (or hydroxylysyl) residues that are exposed to the action of the amine oxidase, it is clear that the action of the enzyme is specifically directed against very selected residues in the peptide chain. Although there is evidence to indicate that some lysyl residues in the helical part of the chains are reactive (ROJKIND et al., 1970), evidence for deamination is best for the non-helical parts of the α-chains. Mention has already been made of the reactive lysyl residue in position 9 in the N-terminal region of the α-chains. More recently, it has been shown that from the C-terminal region of these chains, which is also non-helical, two otherwise identical peptides can be isolated of which one carries a lysyl residue and the other an allysyl (STARK et al., 1971; RAUTERBERG et al., 1972). The

possibility is discussed that the allysyl residue may here serve an intermolecular cross-link.

Cross-links seem not to be unique to the collagen of connective tissue; there has been an observation on cross-links also in the collagen of basement membranes from a variety of locations (TANZER and KEFALIDES, 1973). Thus in these sites also we have to postulate that an amine oxidase of the lysyl oxidase type is active.

B. Observations on Connective Tissue Formation

1. The Role of Copper

Many of the earlier observations on interference with collagen and elastin biosynthesis, made at a time when the function of the connective tissue oxidase was unknown, can be understood on the assumption that for the formation of the cross-links an enzyme of the copper-pyridoxal type is essential.

Studies on the symptoms of copper deficiency in chickens (O'DELL et al., 1961) and in pigs (SHIELDS et al., 1962; WEISSMAN et al., 1963) showed that in the deficient animals there was a defect in the formation of elastic tissues. In particular, the great arteries were affected and death was usually due to aortic aneurism. There have since been a number of studies of the elastin precursors from both pigs (SMITH et al., 1968; SANDBERG et al., 1969, 1971; SMITH et al., 1972) and chickens (ROENSCH et al., 1972); these have not only mechanical properties very different from the finished product, but also differ chemically from elastin: tropoelastin, as the precursor is called, contains about three times as many lysyl residues as elastin, indicating that the oxidative deamination, to produce allysyl, has not taken place. Also, it contains no desmosines and no lysinonorleucine.

For a fuller discussion on the effect of copper deficiencies on connective tissue see CARNES (1968, 1971).

2. The Role of Pyridoxal

If it is true that the enzyme(s) operative in connective tissue belong to the copper-pyridoxal phosphate group, one would expect abnormalities in connective tissue formation in vitamin B_6-deficiency. It has been reported that B_6-deficiency leads to a defect in elastin biosynthesis in the chicken: in elastin from the aorta of B_6-deficient birds the ratio of demosine plus isodesmosine to lysine was specifically reduced (HILL and KIM, 1967; STARCHER, 1969).

There is one pathological condition in which an interaction with the carbonyl group of pyridoxal in the connective tissue enzyme is believed to be the cause of defective formation of both collagen and elastin. The condition is osteolathyrism, a disease seen in experimental animals fed a diet excessively rich in the seeds of certain peas, e.g. the sweet pea (*Lathyrus odoratus*). The toxic compound in the leguminous seeds has been identified as β-(N-γ-L-glutamyl)-aminopropionitrile but under experimental conditions osteolathyrism is usually produced by feeding

β-aminopropionitrile,

$$H_2N \cdot CH_2 \cdot CH_2 \cdot C \equiv N.$$

The compounds used to produce the condition are called lathyrogenic. It should be emphasized, however, that lathyrism, the disease seen in man fed excessive amounts of peas (*Lathyrus sativus*) is a condition with different symptoms, probably caused by a different factor. However, many authors refer to the experimental disease in animals also as lathyrism.

In osteolathyrism the main symptom is an inability to form normal connective tissue and in particular collagen or elastin. There is an extreme mechanical weakness of the connective tissue and deformation of the skeleton, as well as the occurrence of aortic aneurisms. The condition has recently been reviewed (LEVENE, 1971).

It was the work of LEVENE and GROSS (1959) that first suggested that in lathyritic collagen there was an inability of the tropocollagen molecules to associate to form normal macromolecular collagen. Soon afterwards LEVENE (1961, 1962) found that the effect of lathyrogenic compounds could be reversed by pyridoxal and he suggested that a free carbonyl group was essential for the normal formation of collagen; this group, it was suggested, was interfered with by the lathyrogens.

In recent years many authors have used lathyrogenic compounds in order to study the precursors of the connective tissue proteins in the body. With hindsight, it is not surprising that it was found that there was a resemblance between copper deficiency and lathyrism. The ever-growing literature on this subject is fully reviewed by LEVENE (1971). More recently, SYKES and PARTRIDGE (1972) have studied the elastin from the arteries of lathyritic chicks. They were able to extract from a homogenate a soluble protein similar to that found in elastin from copper-deficient animals. The chemical analysis showed that, in contrast to normal insoluble elastin, this protein did not contain any desmosine. On the other hand, the soluble elastin from the lathyritic animals contained 40 lysine residues /1000 total amino-acid residues, whereas normal insoluble elastin contained 4 such residues. A molecular weight of 40–50.00 was assigned to the soluble protein.

PAGE and BENDITT (1967) were among the first to suggest that the lathyrogenic agents exerted their action by inhibiting an amine oxidase of the copper-pyridoxal type. They showed that β-aminopropionitrile was an inhibitor of the pig plasma benzylamine oxidase characterized by BLASCHKO and BUFFONI (1965); the inhibition was competitive and reversible.

That the effect of the lathyrogenic agents is one exerted on the deaminating enzyme is made likely by the observation that lathyrogens prevent the formation of allysyl residues.

It seems probable that the lathyrogenic effect will be found not to be restricted to the compounds that LEVENE (1971) discussed in his review. Now that this condition has been clearly recognized, it will probably also be seen as the consequence of the administration of other compounds capable of interacting with pyridoxal enzymes. One such instances might be quoted as a case in point. THEISS and SCHÄRER (1971) have studied the toxicology of a dopa decarboxylase inhibitor that has been of interest to the students of Parkinson's disease. The inhibitor is N-(DL-seryl)-N^1-(2,3,4-trihydroxybenzyl)-hydrazine (BURKARD et al., 1962).

THEISS and SCHÄRER have found that the daily administration of this compound, also known as Ro4-4602, causes deformations of the skeleton. They have seen that the compound interferes with the normal maturation of fibroblasts, causing a condition accompanied by defective formation of collagen fibrils. Since this compound is bound to interact with pyridoxal enzymes, the possibility should be considered likely that the condition described by THEISS and SCHÄRER is a kind of lathyrism.

Another type of interference with collagen biosynthesis is brought about by the administration of penicillamine. However, the action of penicillamine differs from that of the lathyrogens. The lathyrogens, as inhibitors of the lysyl oxidase, interfere with the enzymic formation of the aldehyde groups essential for cross-linking – this is why there is an excess of lysyl groups – whereas the penicillamine does, at least in moderate doses, not act in the same way: here the lysyl groups are acted upon by the oxidase as in normal animals, but the penicillamine interacts with the aldehyde groups of allysyl (or hydroxyallysyl) residues and thus prevents them from forming the Schiff's base with the amino group of lysyl residues (NIMNI et al., 1970). Only in higher doses does the penicillamine interact also with the aldehyde groups of the enzyme. the aldehyde group in pyridoxal; in other words, it then acts as a lathyrogen (NIMNI et al., 1972).

C. The Amine Oxidases of Connective Tissue

The observations on cross-links in elastic and collagen fibres rest on the assumption that a "lysyl oxidase" of the copper-pyridoxal type is present in the connective tissue.

There have been a number of studies of amine oxidases in connective tissues, but it is not yet known to what extent the observations made are relevant to cross-linking. LOVENBERG et al. (1968) used benzylamine as substrate in observations on preparations from rat and human skin. The oxidation of benzylamine was inhibited by β-aminopropionitrile. However, the question as to the significance of the reaction studied to the problem of "lysyl oxidase" was left open. NAGATSU et al. (1972) achieved a partial purification of an oxidase present in dental pulp; again benzylamine was used as substrate. The oxidation was inhibited by β-aminopropionitrile (see also NARAYANAN et al., 1972), by isoniazid and by cuprizon indicating that a copper-containing enzyme was at work.

The most direct approach to the study of lysyl oxidase was made by PINNELL and MARTIN (1968; see also MARTIN et al., 1970). These authors used as the enzyme a preparation of tibia and femur of 16 day old chicks, and as the substrate a protein from embryonic chick aorta labelled *in vivo* with [3H]6-lysine. The tritium set free when enzyme and substrate were incubated *in vitro* was measured. Tritium release was found to be inhibited by β-aminopropionitrile. The amount of enzyme was found to be at a maximum in cartilage from 17 day old chick embryos and to decrease rapidly after hatching. Lysyl oxidase was absent in chicks that had been placed on a copper-deficient diet for 8 days; this is reminiscent of results on benzylamine oxidase in copper deficiency (BLASCHKO et al., 1965). MARTIN et al. (1970) have found that both elastin and collagen can serve as substrate.

Further observations on the lysyl oxidase from chick aorta have recently been reported by HARRIS et al. (1973), who state that they have achieved a 1000-fold purification of the enzyme. Both soluble and insoluble protein preparations from aorta served as substrates. Gel filtration suggests a molecular weight of the enzyme of about 61000, and this leads to a copper content of somewhat over 1 g atom of Cu per mole.

Two enzyme preparations similar to those used by MARTIN et al. (1970) were prepared by BAILEY and FOWLER (1969) and by FOWLER et al. (1970). These were incubated with the appropriate substrates and, after incubation, the protein substrate was hydrolysed and reduced with tritiated borohydride. In the first experiment (BAILEY and FOWLER, 1969) the enzyme was a soluble preparation prepared from the tibia and femora of 17 day normal chick embryo, and the substrate was an *insoluble* preparation of bones from lathyritic chick embryos. Evidence was obtained of the presence of both aldol and aldimino cross-links in the incubated samples; these were absent in the unincubated samples.

In the second experiment (FOWLER et al., 1970) a similar incubation was carried out in which a *soluble* collagen preparation was used. Here the enzyme, similarly prepared, was made from the tibia and femora of normal 2 week old piglets; the substrate was a soluble protein preparation of Achilles tendon collagen from lathyritic piglets. In the borohydride-reduced hydrolysates of the incubated collagen both syndesine and hydroxylysinonorleucine were demonstrated, whereas they were absent in samples from non-incubated collagen.

Much of the work on connective tissue oxidase has been carried out using conventional substrates of the plasma oxidases. For instance, in chick aorta copper deficiency as well as vitamin B_6 deficiency caused a reduction in the amine oxidase activity of the tissue (KIM and HILL, 1966; HILL and KIM, 1967); these authors used the spectrophotometric method of GORKIN et al. (1962), in which benzylamine serves as substrate. Similarly, in earlier work on the bovine aorta oxidase benzylamine had served as substrate (RUCKER and O'DELL, 1971; HARRIS and O'DELL, 1972). It remains doubtful if these activities are manifestations of the enzyme that is responsible for cross-linking. That a "lysyl oxidase" was present in RUCKER and O'DELL's preparations, at least as one catalyst, is supported by the observation that in addition to the substrates oxidized by the bovine plasma spermine oxidase, the bovine aorta preparation acted also upon lysine-vasopressin, a peptide that contains a lysyl residue (RUCKER and O'DELL, 1971). The authors have discussed the possibility that the plasma oxidase is a secretion product of the connective tissue, and that it represents a different molecular form of the connective tissue enzyme. This possibility is of interest in view of the fact that in several species (e.g., the pig and the goat) the plasma enzymes are absent, or almost absent, at birth, that is to say, at a time when synthesis of protein in the connective tissue takes place at a high rate.

To sum up, from the work just reported, it seems quite possible that the connective tissue contains three amine oxidases: an enzyme related to MAO, secondly, an enzyme closely related to the plasma oxidases (if not identical with them), and, thirdly, a true lysyl oxidase, an enzyme that, like the plasma oxidases, contains copper and a carbonyl group but seems to have a more restricted substrate specificity, acting solely on lysyl (or hydroxylysyl) groups in peptide linkage.

Dr. B. L. O'DELL kindly tells me that this third enzyme is distinguished by its solubility properties, being not extracted by salt buffers or Triton X-100, which does solubilize the second type of oxidase.

The relationship between the plasma oxidases and lysyl oxidase can be resolved only when the connective tissue enzymes are fully purified. In order to obtain some information on possible biochemical relationships between the two groups of enzymes, a new approach was chosen by BUFFONI and HOPE (unpublished); see also BUFFONI and DELLA CORTE (1972). A rabbit antibody against highly purified, re-crystallized benzylamine oxidase of pig plasma was prepared and this antibody was used to locate by immunofluorescence microscopy tissue constituents that would cross-react with the antibody. Newborn piglets were chosen for this study, since it is known that at birth there is practically no benzylamine oxidase activity present in the plasma (BLASCHKO, 1962; BLASCHKO and BONNEY, 1962). All the tissues tested showed specific fluorescence; these were: the media and adventitia of the aorta and other arteries, the connective tissue in uterus, kidneys and tendons.

The immunofluorescence technique indicates that the connective tissue of the newborn pig contains a protein that cross-reacts with the antibody against plasma benzylamine oxidase, at a time when there is practically no plasma oxidase present. In view of our knowledge that the tissues contain an enzyme capable of acting upon benzylamine, the close immunological relationship between a tissue component and plasma benzylamine oxidase becomes particularly interesting. It is difficult to escape the conclusion that the antigen shown up by immunofluorescence microscopy in the connective tissue must be closely related to the catalyst that is responsible for the reaction occurring prior to the formation of the cross-links.

VI. Final Comments

The study of the amine oxidases has for many years been focussed upon the biogenic amines, the catecholamines, 5-HT and histamine. One of the aims of this review has been to show that the field of interest in amine oxidases has widened in recent years. In animal biochemistry and physiology, the study of the polyamines is slowly beginning to find a place, three hundred years after Antony van Leeuwenhoek's discovery of spermine. To what extent enzymes like spermine oxidase are concerned in the catabolism of endogenous polyamines is still obscure, but most probably with the growing interest in the area research will soon concern itself with these problems.

The most unexpected new development of the past decade is the growing awareness of the importance of amine oxidases in the biosynthesis of connective tissue macromolecules. It has been one of the aims of this review to relate the recent work in this field to the earlier studies on amine oxidases. The questions that arise from the new work are whether there are more than one enzyme at work, e.g. one for the collagen fibres and one for elastin. Another most interesting problem will be that of the site of action of these enzymes. Is the lysyl oxidase

operative in the connective tissue cells or extracellularly, after the precursor proteins have been extruded?

These questions are also of relevance to the problem of the amine oxidases of mammalian blood plasma. These enzymes still present a challenge. They have been important in the study of the copper-containing enzymes in general, because they could be relatively readily purified and crystallized, and in many respects they have served as prototypes, in the study of DAO and also of the connective tissue enzymes. Their own biological significance, however, is uncertain.

The plasma enzymes act on many substances that are also substrates of either MAO and DAO. Is the disposal of these substances their only, or main, function? Similarly, is the close relationship between plasma oxidases and lysyl oxidase indicative of a function of the latter enzyme in relation to known substrates of the plasma enzymes (spermine, histamine, mescaline, dopamine) or is activity in the connective tissue on these amines due to contamination with another enzyme? These are all questions to which answers will be obtained by further experimentation.

Altogether, the copper-containing oxidases are less well studied by the methods that have proved useful in research on MAO. We would like to know more on the intracellular localization of DAO. The study of the localization of MAO has been fruitful for our understanding of the biological functions of this enzyme.

The study of MAO also poses many new and intriguing questions. What is the physiological significance of MAO in animals like the insects where so many interesting substrates of the enzyme have been found?

Another most interesting new development is the study of the isoenzymes of MAO. There can be no doubt that functionally different kinds of MAO can be distinguished, and it can be hoped that future work will settle the question whether the differences in substrate specificity are due to different molecular species of protein, possibly genetically determined, or to different activities of one enzyme, determined by different environment, e.g. in a membrane.

There has also been a recent report on isoenzymes of the human plasma amine oxidase (RAUCH and RAUCH, 1973). The author has recently speculated on the possibility that in the nervous system MAO may have different functions in different locations (BLASCHKO, in press). Possibly even in one neurone MAO may act in different locations. If we have to distinguish between receptor-related MAO and nerve-ending MAO it is legitimate to enquire if these two kinds of oxidase are similar or whether they are different. This is just one aspect of the new discoveries that remains to be exploited.

Another problem, scarcely dealt with in this review, will become important in the near future. Much has been learnt of the regulatory mechanisms that operate in amine biosynthesis, but much less is at present known of the influences that act upon the turnover of the classical MAO.

The subject matter discussed in this review has been greatly determined by the writer's own research interests. This is why many of the problems related to function of the enzymes have been more extensively dealt with than the molecular aspects. There is little doubt, however, that the study of the latter will be greatly extended in the near future, so that some of the problems here discussed will be resolved.

Altogether, there is little reason to doubt that the study of the amine oxidases will continue to present the experimenter with new and rewarding problems to solve.

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References

ADACHI, O., YAMADA, H.: Amine oxidases of microorganisms. VIII. Purification and properties of amine oxidase of Fusarium culmorum. Memoirs of the Research Institute for Food Science, Kyoto University **31**, 10–18 (1970).

ADACHI, O., YAMADA, H., OGATA, K.: Purification and properties of putrescine oxidase of *Micrococcus rubens*. Agricult. biol. Chem. **30**, 1202–1210 (1966).

ADLER-GRASCHINSKY, E., LANGER, S. Z., RUBIO, M. C.: Metabolism of norepinephrine released by phenoxybenzamine in isolated guinea-pig atria. J. Pharmacol. exp. Ther. **180**, 286–301 (1972).

ALLES, G. A., HEEGAARD, E. V.: Substrate specificity of amine oxidase. J. biol. Chem. **147**, 487–503 (1943).

ARGENTO-CERU, M. P., SARTORI, C., AIUTUORI, F.: Diamine oxidase in rabbit liver. 1. Assay conditions and subcellular localization. Europ. J. Biochem. **34**, 369–374 (1973a).

ARGENTO-CERU, M. P., SARTORI, C., AIUTUORI, F.: Diamine oxidase in rabbit liver. 2. Submicroscopial distribution. Europ. J. Biochem. **34**, 375–382 (1973b).

ARMSTRONG, M. D., MCMILLAN, A., SHAW, K. N.: 3-Methoxy-4-hydroxy-D-mandelic acid, a urinary metabolite of norepinephrine. Biochim. biophys. Acta (Amst.) **25**, 422–423 (1957).

ARUNLAKSHANA, O., MONGAR, J. L., SCHILD, H. O.: Potentiation of pharmacological effects of histamine by histaminase inhibitors. J. Physiol. (Lond.) **123**, 32–54 (1954).

AVAKIAN, V. M., CALLINGHAM, B. A.: An effect of adrenalectomy upon catecholamine metabolis. Brit. J. Pharmacol. **33**, 211–212P (1968).

AVAKIAN, O. V., GILLESPIE, J. S.: Uptake of noradrenaline by adrenergic nerves, smooth muscle and connective tissue in isolated perfused arteries and its correlation with the vasoconstrictor response. Brit. J. Pharmacol. **32**, 168–184 (1968).

AXELROD, J.: The metabolism of catecholamines *in vivo* and *in vitro*. Pharmacol. Rev. **11**, 402–408. (1959).

BACHRACH, U.: Spermidine oxidase from *Serratia marcescens*. J. biol. Chem. **237**, 3443–3448 (1962).

BACHRACH, U.: Metabolism and function of spermine and related polyamines. Ann. Rev Microbiol. **24**, 109–134 (1970).

BAILEY, A. J., FOWLER, L. J.: *In vitro* enzymic biosynthesis of two inter-chain crosslinks of bone collagen. Biochem. biophys. Res. Commun. **35**, 672–675 (1969).

BAILEY, A. J., PEACH, C. M.: The chemistry of the collagen cross-links. The absence of reduction of dehydrolysinonorleucine and dehydrohydroxylysinonorleucine *in vivo*. Biochem. J. **121**, 257–259 (1971).

BAILEY, A. J., PEACH, C. M., FOWLER, L. J.: Chemistry of the collagen cross-links. Isolation and characterization of two intermediate intermolecular cross-links in collagen. Biochem. J. **117**, 819–831 (1970a).

BAILEY, A. J., PEACH, C. M., FOWLER, L. J.: The biosynthesis of intermolecular cross-links in collagen. In: E. A. BALAZS (Ed.): Chemistry and molecular biology of the intercellular matrix, Vol. 1, p. 385–404. London and New York: Academic Press 1970.

BANKS, P., MAYOR, D.: Intra-axonal transport in noradrenergic neurons in the sympathetic nervous system. Biochem. Soc. Symp. **36**, 133–149 (1972).

BARDSLEY, W. G., CRABBE, M. J. C., SHINDLER, J. S.: Kinetics of the diamine oxidase reaction. Biochem. J. **131**, 459–469 (1973).

BARDSLEY, W. G., CRABBE, M. J. C., SHINDLER, J. S., ASHFORD, J. S.: Oxidation of p-dimethylaminomethylbenzylamine by pig kidney diamine oxidase. Biochem. J. **127**, 875–879 (1972).

BARDSLEY, W. G., HILL, C. M., LOBLEY, R. W.: A reinvestigation of the substrate specificity of pig kidney diamine oxidase. Biochem. J. **117**, 169–176 (1970).

BARKER, D. L., MOLINOFF, P. B., KRAVITZ, E. A.: Octopamine in the lobster nervous system. Nature New Biology **236**, 61–63 (1972).

BARLOW, R. B., BLASCHKO, H., HIMMS, J. M., TRENDELENBURG, U.: Observations on ω-aminopolymethylene trimethylammonium compounds. Brit. J. Pharmacol. **10**, 116–123 (1955).

BARNES, M. J., CONSTABLE, B. J., MORTON, L. F., KODICEK, E.: Hydroxylysine in the N-terminal regions of the α_1- and α_2-chains of various collagens. Biochem. J. **125**, 433–437 (1971).

BARONDES, S. H.: On the site of synthesis of the mitochondrial protein of nerve endings. J. Neurochem. **13**, 721–727 (1966).

BAUDHUIN, P., BEAUFAY, H., RAHMAN-LI, Y., SELLINGER, O. Z., WATTIAUX, R., JACQUES, P., DEDUVE, C.: Tissue fractionation studies 17. Intracellular distribution of monoamine oxidase, aspartate aminotransferase, alanine aminotransferase, D-amino acid oxidase and catalase in rat liver tissue. Biochem. J. **92**, 179–184 (1964).

BAUMGARTEN, H. G., BJÖRKLUND, A., LACHENMAYER, L., NOBIN, A., ROSENGREN, E.: Evidence for the existence of serotonin-, dopamine-, and noradrenaline-containing neurones in the gut of *Lampetra fluviatilis*. Z. Zellforsch. **141**, 33–54 (1973).

BAXTER, R.: Origin and continuity of mitochondria. In: Origin and continuity of cell organelles (J. REINERT, H. URSPRUNG, Eds.), p. 46–64. Berlin-Heidelberg-New York: Springer 1971.

BAXTER-GRILLO, D. L.: Enzyme histochemistry and hormones of the developing gastrointestinal tract of the chick embryo III. Enterochromaffin cells—their possible products, glucagon, 5-hydroxytryptamine and the reaction of monoamine oxidase. Histochemie **21**, 129–135 (1970).

BEATTIE, D. S.: Enzyme localization in the inner and outer membrane of rat liver mitochondria. Biochem. biophys. Res. Commun. **31**, 901–907 (1968).

BEATTIE, D. S., BASFORD, R. E., KORITZ, S. B.: The inner membrane as the site of the in vitro incorporation of L-[^{14}C]leucine into mitochondrial protein. Biochemistry **6**, 3099–3106 (1967).

BERTACCINI, G.: Discussion Remark. In: Regional Neurochemistry (S. S. KETY, J. ELKES, Eds.), p. 305–306. Oxford-London-New York-Paris: Pergamon Press 1961.

BEST, C. H.: The disappearance of histamine from autolysing lung tissue. J. Physiol. (Lond.) **67**, 256–263 (1929).

BEST, C. H., MCHENRY, E. W.: The inactivation of histamine. J. Physiol. (Lond.) **70**, 349–372 (1930).

BLACK, J. W., DUNCAN, W. A. M., DURANT, C. J., GANELLIN, C. R., PARSONS, E. M.: Definition and antagonism of histamine-H_2 receptors. Nature **236**, 385–390 (1972).

BLASCHKO, H.: The specific action of l-dopa decarboxylase. J. Physiol. (Lond.) **96**, 50–51P (1939).

BLASCHKO, H.: Amine oxidase in *Sepia officinalis*. J. Physiol. (Lond.) **99**, 364–369 (1941).

BLASCHKO, H.: Amine oxidase and amine metabolism. Pharmacol. Rev. **4**, 415–458 (1952a).

BLASCHKO, H.: Enzymic oxidation of 5-hydroxytryptamine in mammalian and cephalopod tissues. Biochem. J. **52**, X (1952b).

BLASCHKO, H.: Oxidation of 1,4-methylhistamine by mammalian plasma. J. Physiol. (Lond.) **148**, 570–573 (1959).

BLASCHKO, H.: Primary and secondary amines as substrates of amine oxidases. J. Physiol. (Lond.) **153**, 17–18P (1960).

BLASCHKO, H.: The amine oxidases of mammalian blood plasma. Advances in comparative Physiology and Biochemistry **1**, 67–116 (1962).

BLASCHKO, H.: Introduction and historical background. Advances in biochem. Psychopharmacol. **5**, 1–10 (1972).

BLASCHKO, H.: Introductory remarks on monoamine oxidase. In: Catecholamines and their Enzymes in the Neuropathology of Schizophrenia (S. S. KETY, S. W. MATTHYSSE, Eds.). In print.

BLASCHKO, H., BOADLE, M. C.: Substrate specificity of amine oxidases. In: Pyridoxal Catalysis: Enzymes and Model Systems (SNELL, E. E., BRAUNSTEIN, A. E., SEVERIN, E. S., TORCHINSKY, YU. M., Eds.), p. 339–346. New York-London-Sydney: Interscience 1968.

BLASCHKO, H., BOADLE, M. C., STRICH, S. J.: Enzymic oxidation of amines in cyclostomes. J. Physiol. (Lond.) **204**, 104–105P (1969).

BLASCHKO, H., BONNEY, R.: Spermine oxidase and benzylamine oxidase. Distribution, development and substrate specificity. Proc. roy. Soc. B **156**, 268–279 (1962).

BLASCHKO, H., BUFFONI, F.: Pyridoxal phosphate as a constituent of the histaminase of pig plasma. Proc. roy. Soc. B **163**, 45–60 (1965).

BLASCHKO, H., BUFFONI, F., WEISSMAN, N., CARNES, W. H., COULSON, W. F.: The amine oxidase of pig plasma in copper deficiency. Biochem. J. **96**, 4C–5C (1965).

BLASCHKO, H., CHATTERJEE, M. L., HIMMS, J. M., ALBERT, A.: Enzymic oxidation of derivatives of ethylenediamine. Brit. J. Pharmacol. **10**, 314–316 (1955).

BLASCHKO, H., CHRUSCIEL, T. L.: Observations on the substrate specificity of amine oxidases. Brit. J. Pharmacol. **14**, 364–367 (1959).

BLASCHKO, H., COLHOUN, E. H., FRONTALI, N.: Occurrence of amine oxidase in an insect, *Periplaneta americana L.* J. Physiol. (Lond.) **156**, 28P (1961).

BLASCHKO, H., DUTHIE, R.: Substrate specificity of amine oxidases. Biochem. J. **39**, 478–481 (1945).

BLASCHKO, H., HAWES, R.: Observations on spermine oxidase of mammalian plasma. J. Physiol. (Lond.) **145**, 124–131 (1959).

BLASCHKO, H., HAWKINS, J.: Enzymic oxidation of aliphatic diamines. Brit. J. Pharmacol. **5**, 625–632 (1950).

BLASCHKO, H., HAWKINS, J.: Observations on amine oxidase in cephalopods. J. Physiol. (Lond.) **118**, 88–93 (1952).

BLASCHKO, H., HELLMANN, K.: Pigment formation from tryptamine and 5-hydroxytryptamine in tissues: a contribution to the histochemistry of amine oxidase. J. Physiol. (Lond.) **122**, 419–427 (1953).

BLASCHKO, H., HIMMS, J. M.: Amine oxidase in the earthworm. J. Physiol. (Lond.) **120**, 445–448 (1953).

BLASCHKO, H., HIMMS, J. M.: Enzymic oxidation in Decapods. J. exp. Biol. **31**, 1–7 (1954).

BLASCHKO, H., HOPE, D. B.: Observations on the distribution of amine oxidase in Invertebrates. Arch. Biochem. **69**, 10–15 (1957).

BLASCHKO, H., KIRPEKAR, S. M., PHIPPS, S.: Observations on the enzymic oxidation of ω-amino acids. Arch. int. Pharmacodyn. **139**, 120–122 (1962).

BLASCHKO, H., KURZEPA, S.: Potentiation of the response to histamine by picolylamines. Brit. J. Pharmacol. **19**, 544–551 (1962).

BLASCHKO, H., LEVINE, W. G.: Metabolism of indolealkylamines. In: Handbuch der experimentellen Pharmakologie, Vol. 19 (V. ERSPAMER, Ed.), p. 212–244. Berlin-Heidelberg-New York: Springer 1965.

BLASCHKO, H., RICHTER, D., SCHLOSSMANN, H.: The inactivation of adrenaline. J. Physiol. (Lond.) **90**, 1–19 (1937a).

BLASCHKO, H., RICHTER, D., SCHLOSSMANN, H.: The oxidation of adrenaline and other amines. Biochem. J. **31**, 2187–2196 (1937b).

BLOOM, F. E., SIMS, K. L., WEITSEN, H. A., DAVIS, G. A., HANKER, J. S.: Cytochemical differentiation between monoamine oxidase and other neuronal oxidases. Advances in biochem. Psychopharmacol. **5**, 243–262 (1972).

BOADLE, M. C.: A comparative study of amine oxidases. D. Phil. Thesis, Oxford 1967.

BOADLE, M. C.: Observations on a histaminase of invertebrate origin: a contribution to the study of cephalopod amine oxidases. Comp. Biochem. Physiol. **30**, 611–620 (1969).

BOADLE, M. C., BLASCHKO, H.: Cockroach amine oxidase: classification and substrate specificity. Comp. Biochem. Physiol. **25**, 129–138 (1968).

BOADLE, M. C., BLOOM, F. E.: A method for the fine structural demonstration of monoamine oxidase activity. J. Histochem. Cytochem. **17**, 331–340 (1969).

BORN, G. V. R.: 5-Hydroxytryptamine receptors. In: Smooth Muscle (BÜLBRING, E., BRADING, A. F., JONES, A. W., TOMITA, T., Eds.), p. 418–450. London: Arnold 1970.

BORNSTEIN, P., PIEZ, K. A.: The nature of the intramolecular cross-links in collagen. The separation and characterization of peptides from the cross-link region of rat skin collagen. Biochemistry (Easton) **5**, 3460–3473 (1966).

BRUNNER, G., BYGRAVE, F. L.: Microsomal marker enzymes and their limitations in distinguishing the outer membrane of rat liver mitochondria from the microsomes. Europ. J. Biochem. **8**, 530–534 (1969).

BRUNNER, G., NEUPERT, W.: Turnover of outer and inner membrane proteins of rat liver mitochondria. FEBS Letters **1**, 153–155 (1968).

BUFFONI, F.: Histaminase and related amine oxidases. Pharmacol. Rev. **18**, 1163–1199 (1966).

BUFFONI, F.: Pyridoxal catalysis in pig plasma benzylamine oxidase (histaminase). In: Pyridoxal catalysis: Enzymes and model systems (SNELL, E. E., BRAUNSTEIN, A. E., SEVERIN, E. S., TORCHINSKY, YU. M., Eds.), p. 363–374. New York-London-Sydney: Interscience 1968.

BUFFONI, F., BLASCHKO, H.: Benzylamine oxidase and histaminase: purification and crystallization of an enzyme from pig plasma. Proc. roy. Soc. B **161**, 153–167 (1964).

BUFFONI, F., DELLA CORTE, L.: Pig plasma benzylamine oxidase. Advances in biochem. Psychopharmacol. **5**, 133–149 (1972).

BUFFONI, F., DELLA CORTE, L., IGNESTI, G.: Pig plasma benzylamine oxidase (histaminase): Kinetic studies. Pharmacol. Res. Commun. **4**, 99–122 (1972).

BUFFONI, F., DELLA CORTE, L., KNOWLES, P. F.: The nature of copper in pig plasma benzylamine oxidase. Biochem. J. **106**, 575–576 (1968).

BURKARD, W. P., GEY, K. F., PLETSCHER, A.: A new inhibitor of decarboxylase of aromatic amino acids. Experientia (Basel) **18**, 411–412 (1962).

BURKARD, W. P., GEY, K. F., PLETSCHER, A.: Diamine oxidase in the brain of vertebrates. J. Neurochem. **10**, 183–186 (1963).

BURN, J. H.: The enzyme at sympathetic nerve endings. Brit. med. J. **1952 I**, 784–785.

BYGRAVE, F. L., BÜCHER, TH.: Synthesis *in vivo* of the lecithin component of the inner and outer membranes of rat liver mitochondria. FEBS Letters **1**, 42–45 (1968).

CAESAR, P. M., COLLINS, G. G. S., SANDLER, M.: Catecholamine metabolism and monoamine oxidase activity in adrenalectomized rats. Biochem. Pharmacol. **19**, 921–926 (1970).

CALLINGHAM, B. A., DELLA CORTE, L.: Effect of adrenalectomy upon some rat heart enzymes. Brit. J. Pharmacol. **41**, 392P (1971).

CALLINGHAM, B. A., DELLA CORTE, L.: The influence of growth and of adrenalectomy upon some rat heart enzymes. Brit. J. Pharmacol. **46**, 530–531 (1972).

CAMPELLO, A. P., TABOR, C. W., TABOR, H.: Resolution of spermidine dehydrogenase from *Serratia marcescens:* requirements for flavin adenin dinucleotide and an additional electron carrier. Biochem. biophys. Res. Commun. **19**, 6–9 (1965).

CARLSSON, A.: Pharmacological depletion of catecholamine stores. Pharmacol. Rev. **18**, 541–549 (1966).

CARLSSON, A., BÉDARD, P., LINDQVIST, M., MAGNUSSON, T.: The influence of nerve-impulse flow on the synthesis and metabolism of 5-hydroxytryptamine in the central nervous system. Biochem. Soc. Symp. **36**, 17–32 (1972).

CARLSTEN, A.: Effect of adrenalectomy on lymph and plasma histaminase. Acta physiol. scand. **20**, suppl. **70**, 33–46 (1950).

CARNES, W. H.: Copper and connective tissue metabolism. International Review of Connective Tissue Research **4**, 197–232 (1968).

CARNES, W. H.: Role of copper in connective tissue metabolism. Fed. Proc. **30**, 995–1000 (1971).

CHAUDHARY, K. D., SRIVASTAVA, U., LEMONDE, J. A.: Monoamine oxidase in *Tribolium confusum* Duval (Coleoptera). Biochim. biophys. Acta (Amst.) **132**, 290–299 (1967).

CODE, C. F., CODY, D. T., HURN, M., KENNEDY, J. C., STRICKLAND, M.: The simultaneous release of histamine and a histamine-destroying factor during anaphylaxis in rats. J. Physiol. (Lond.) **156**, 207–216 (1961).

COLLINS, G. G. S., SANDLER, M.: Human blood platelet monoamine oxidase. Biochem. Pharmacol. **20**, 289–296 (1971).

CONSOLO, S., GIACOBINI, E., KARJALAINEN, K.: Monoamine oxidase: sympathetic ganglia of the cat. Acta physiol. scand. **74**, 513–520 (1968).

COSTA, E., SANDLER, M. (Eds.): Monoamine oxidases — new vistas. Advances in biochem. Psychopharmacol. **5**, 1–454 (1972).

COSTA, T. M., ROTILIO, G., FINAZZI-AGRO, A., VALLOGINI, M. P., MONDOVI, B.: On the active site of diamine oxidase: kinetic studies. Arch. Biochem. **147**, 8–13 (1971).

COTZIAS, G. L., DOLE, V. P.: Metabolism of amines II. Mitochondrial localization of monoamine oxidase. Proc. Soc. exp. Biol. (N.Y.) **78**, 157–160 (1951).

DAHLBÄCK, O., HANSSON, R., TIBBLING, G., TRYDING, N.: The effect of heparin on diamine oxidase and lipoprotein lipase in human lymph and blood plasma. Scand. J. clin. Lab. Invest. **21**, 17–25 (1968).

DAHLSTRÖM, A.: Axoplasmic transport (with particular respect to adrenergic neurons). Phil. Trans. B **261**, 325–358 (1971).

DAHLSTRÖM, A.: The axonal transport of monoamine oxidases. Advances in biochem. Psychopharmacol. **5**, 293–305 (1972).

DE LA LANDE, I. S., JELLETT, L. B.: Relationship between the roles of monoamine oxidase and sympathetic nerves in the vasoconstrictor response of the rabbit ear artery to norepinephrine. J. Pharmacol. exp. Ther. **180**, 47–55 (1972).

DELLA CORTE, L., PERRINO, I.: Il ruolo delle aminossidasi plasmatiche nel metabolismo della dopamina. Farmaco, Ed. sci. **23**, 204–209 (1968).

DE SA, R. J.: Putrescine oxidase from *Micrococcus rubens*, Purification and properties of the enzyme. J. biol. Chem. **247**, 5527–5534 (1972).

DRASKOCZY, P. R., TRENDELENBURG, U.: Intraneuronal and extraneuronal accumulation of sympathomimetic amines in the isolated nictitating membrane of the cat. J. Pharmacol. exp. Ther. **174**, 290–306 (1970).

EADY, R. R., JARMAN, T. R., LARGE, P. J.: Microbial oxidation of amines. Partial purification of a mixed-function secondary amine oxidase system from *Pseudomonas aminovorans* that contains an enzymically active cytochrome—P-420-type haemoprotein. Biochem. J. **125**, 449–459 (1971).

EADY, R. R., LARGE, P. J.: Microbial oxidation of amines. Spectral and kinetic properties of primary amine dehydrogenase of Pseudomonas AM1. Biochem. J. **123**, 757–771 (1971).

EHINGER, B., FALCK, B.: Concomitant adrenergic and parasympathetic fibres in the rat iris. Acta physiol. scand. **67**, 201–207 (1966).

EHINGER, B., FALCK, B., ROSENGREN, A.-M., SPORRONG, B.: Acetylcholine in adrenergic nerve terminals of the cat iris. J. Physiol. (Lond.) **209**, 557–565 (1970).

EIDUSON, S.: Ontogenetic development of monoamine oxidase. Advances in biochem. Psychopharmacol. **5**, 271–287 (1972).

EISENFELD, A. J., AXELROD, J., KRAKOFF, L.: Inhibition of the extraneuronal accumulation and metabolism by adrenergic blocking agents. J. Pharmacol. exp. Ther. **156**, 107–113 (1967a).

EISENFELD, A. J., LANDSBERG, L., AXELROD, J.: Effect of drugs on the accumulation and metabolism of extraneuronal norepinephrine in the rat heart. J. Pharmacol. exp. Ther. **158**, 378–385 (1967b).

ELIASSEN, K. A.: Metabolism of ^{14}C-histamine in goats and pigs treated with aminoguanidine. Acta physiol. scand. **88**, 1–7 (1973).

ERÄNKÖ, L., ERÄNKÖ, O.: Effect of hydrocortisone on histochemically demonstrable catecholamines in the sympathetic ganglia and extraneuronal chromaffin tissue of the rat. Acta physiol. scand. **84**, 125–133 (1972).

ERÄNKÖ, O., ERÄNKÖ, L., HILL, C. E., BURNSTOCK, G.: Hydrocortisone-induced increase in the number of small intensely fluorescent cells and the histochemically demonstrable catecholamine contact in cultures of sympathetic ganglia of the rat. Histochem. J. **4**, 49–58 (1972).

ERSPAMER, V.: Identification of octopamine as 1-p-hydroxy-phenylethanolamine. Nature (Lond.) **169**, 800–801 (1952).

ERWIN, V. G., HELLERMAN, L.: Mitochondrial monoamine oxidase I. Purification and characterization of the bovine kidney enzyme. J. biol. Chem. **242**, 4230–4238 (1967).

ERWIN, V. G., SIMON, R. J.: Occurrence of newly synthesized monoamine oxidase in subcellular fractions of the rat liver. J. pharm. Sci. **58**, 1033–1035 (1969).

FINAZZI-AGRO, A., ROTILIO, G., COSTA, T. M., MONDOVI, B.: Evidence for a ping-pong mechanism in the diamine oxidase reaction. FEBS Letters **4**, 31–32 (1969).

FISCHER, J. E., KOPIN, I. J., AXELROD, J.: Evidence for extraneuronal binding of norepinephrine. J. Pharmacol. exp. Ther. **147**, 181–185 (1965).

FOWLER, L. J., PEACH, C. M., BAILEY, A. J.: *In vitro* studies on the enzymic biosynthesis of the collagen crosslinks. Biochem. biophys. Res. Commun. **41**, 251–259 (1970).

FRANZBLAU, C., FARIS, B., LENT, R. W., SALCEDO, L. L., SMITH, B., JAFFE, R., CROMBIE, G.: Chemistry and biosynthesis of crosslinks in elastin. In: Chemistry and Molecular Biology of the Intercellular Matrix (BALAZS, E. A., Ed.), Vol. I, p. 617–639. London-New York: Academic Press 1970.

FRANZBLAU, C., SINEX, F. M., FARIS, B., LAMPIDIS, R.: Identification of a new crosslinking amino acid in elastin. Biochem. biophys. Res. Commun. **21**, 575–581 (1965).

FREYBURGER, W. A., GRAHAM, B. E., RAPPORT, M. M., SEAY, P. H., GOVIER, W. M., SWOAP, O. F., VAN DER BROOK, M. J.: J. Pharmacol. exp. Ther. **55**, 80–86 (1952).

FUJIWARA, M., TANAKA, C., HIKOSAKA, H., OKEGAWA, T.: Cytological localization of noradrenaline, monoamine oxidase and acetylcholinesterase in salivary glands of dog. J. Histochem. Cytochem. **14**, 483–490 (1966).

FULLER, R. W.: Selective inhibition of monoamine oxidase. Advances in biochem. Psychopharmacol. **5**, 339–354 (1972).

FURNESS, J. B., COSTA, M.: Monoamine oxidase histochemistry of enteric neurones in the guinea pig. Histochemie **28**, 324–336 (1971).

FURNESS, J. B., COSTA, M.: The nervous release and the action of substances which affect intestinal muscle through neither adrenoreceptors nor cholinoreceptors. Phil. Trans. B **265**, 123–133 (1973).

FUXE, K., HÖKFELT, T., UNGERSTEDT, U.: Localization of indolealkylamine in CNS. Advances Pharmacol. **6A**, 235–251 (1968).

GALLOP, P. M., BLUMENFELD, O. O., HENSON, E., SCHNEIDER, A. L.: Isolation and identification of α-amino-aldehydes in collagen. Biochemistry **7**, 2409–2430 (1968).

GALLOP, P. M., BLUMENFELD, O. O., PAZ, M. A.: Aldehydes and aldehyde derivatives in collagen. In: Chemistry and molecular biology of the intercellular matrix (BALAZS, E. A., Ed.), Vol. 1, p. 179–194. London-New York: Academic Press 1970.

GEAR, A. R. L.: Inner- and outer-membrane enzymes of mitochondria during liver regeneration. Biochem. J. **120**, 577–587 (1970).

GHISLA, S., HEMMERICH, P.: Synthesis of the flavocoenzyme of monoamine oxidase. FEBS Letters **16**, 229–232 (1971).

GIACOBINI, E., KERPEL-FRONIUS, S.: Histochemical and biochemical correlations of monoamine oxidase activity in autonomic and sensory ganglia of the cat. Acta physiol. scand. **78**, 522–528 (1970).

GILLESPIE, J. S., HAMILTON, D. N. H., HOSIE, R. J. A.: The extraneuronal uptake and localization of noradrenaline in the cat spleen and the effect on this of some drugs, of cold and of denervation. J. Physiol. (Lond.) **206**, 563–590 (1970).

GILLESPIE, J. S., MUIR, T. C.: Species and tissue variation in extraneuronal and neuronal accumulation of noradrenaline. J. Physiol. (Lond.) **206**, 591–604 (1970).

GLENNER, G. G., BURTNER, H. J., BROWN, G. W., JR.: The histochemical demonstration of monoamine oxidase activity by tetrazolium salts. J. Histochem. Cytochem. **5**, 591–600 (1957).

GOMES, B., IGAUE, I., KLOEPFER, H. G., YASUNOBU, K. T.: Amine oxidase XIV. Isolation and characterization of the multiple forms of beef liver amine oxidase components. Arch. Biochem. **132**, 16–27 (1969).

GORIDIS, C., NEFF, N. H.: Evidence for a specific monoamine oxidase associated with sympathetic nerves. Neuropharmacology **10**, 557–564 (1971a).

GORIDIS, C., NEFF, N. H.: Monoamine oxidase in sympathetic nerves: a transmitter specific enzyme type. Brit. J. Pharmacol. **43**, 814–818 (1971b).

GORKIN, V. Z.: Qualitative alterations in enzymatic properties of amine oxidases. Advances in biochem. Psychopharmacol. **5**, 55–65 (1972).

GORKIN, V. Z., GRIDNEVA, L. I., ROMANOVA, L. A., SEVERINA, I. S.: Spectrophotometric determination of monoamine oxidase activity in mitochondria. Biochemistry **27**, 852–860 (1962).

GORYACHENKOVA, E. V., SHERBATIUK, L. I., ZAMARAEV, C. I.: Purification of pig kidney diamine oxidase and an investigation of the nature of its prosthetic groups. In: Pyridoxal Catalysis: Enzymes and model systems (SNELL, E. E., BRAUNSTEIN, A. E., SEVERIN, E. S., TORCHINSKY, YU. M., Eds.), p. 391–402. New York-London-Sydney: Interscience 1968.

HAGEN, P., WEINER, N.: Enzymic oxidation of pharmacologically active amines. Fed. Proc. **18**, 1005–1012 (1959).

HAKANSON, R., OWMAN, C.: Pineal decarboxylase and monoamine oxidase activities as related to the monoamine stores. J. Neurochem. **13**, 597–605 (1966).

HAMILTON, G. A.: A possible model reaction for pyridoxal phosphate dependant amine oxidases. In: Pyridoxal catalysis: Enzymes and model systems (SNELL, E. E., BRAUNSTEIN, A. E., SEVERIN, E. S., TORCHINSKY, YU. M., Eds.), p. 375–390. New York-London-Sydney: Interscience 1968.

HANKER, J. S., KUSYK, C. J., BLOOM, F. E., PEARSE, A. G. E.: The demonstration of dehydrogenases and monoamine oxidase by the formation of osmium blacks at the sites of Hatchett's brown. Histochemie **33**, 205–230 (1973).

HANNONEN, P., JÄNNE, J., RAINA, A.: Partial purification and characterization of spermine synthase from rat brain. Biochim. biophys. Acta (Amst.) **289**, 225–231 (1972a).

HANNONEN, P., RAINA, A., JÄNNE, J.: Polyamine synthesis in the regenerating rat liver: stimulation of S-adenosyl-methionine decarboxylase and spermidine and spermine syntheses after partial hepatectomy. Biochim. biophys. Acta (Amst.) **273**, 84–90 (1972b).

HANSSON, R., THYSELL, H.: Diamine oxidase in blood plasma in some vertebrates and *Anodonta cygnea* before and after injection of heparin. Acta physiol. scand. **74**, 533–542 (1968).

HARE, M. L. C.: Tyramine oxidase. I. A new enzyme system in liver. Biochem. J. **22**, 968–979 (1928).

HARRIS, E. D., GONNERMAN, W. A., O'DELL, B. L.: Purification properties of chick aorta lysyl oxidase. Fed. Proc. **32**, 594 (1973).

HARRIS, E. D., O'DELL, B. L.: Comparison of soluble and particulate amine oxidases from bovine aorta. Biochim. biophys. Res. Commun. **48**, 1173–1178 (1972).

HARTMAN, B. K., UDENFRIEND, S.: The use of immunological techniques for the characterization of bovine monoamine oxidase from liver and brain. Advances in biochem. Psychopharmacol. **5**, 119–128 (1972).

HARTMAN, B. K., YASUNOBU, K. T., UDENFRIEND, S.: Immunological identity of the multiple forms of beef liver mitochondrial monoamine oxidase. Arch. Biochem. **147**, 797–804 (1971).

HAWKINS, J.: The localization of amine oxidase in the liver cell. Biochem. J. **50**, 577–581 (1952a).

HAWKINS, J.: Amine oxidase activity of rat liver in riboflavin deficiency. Biochem. J. **51**, 399–404 (1952b).

HAWKINS, J., WALKER, J. M.: The effect of colchicine on the enzyme content of regenerating rat liver and on the pressor amine content of the adrenal. Brit. J. Pharmacol. **7**, 152–160 (1952).

HAYES, D. K., WASH, D. B., ODESSER, D. B., SCHECHTER, M. S.: Phosphodiesterase and monoamine oxidase in larvae of the European corn borer exposed to two photoperiodic regimes. Fed. Proc. **30**, 1268 Abs (1971).

HAYES, D. K., WASH, D. B., SCHECHTER, M. S.: Monoamine oxidase activity in larvae of the European corn borer. J. econ. Entomol. **65**, 1229–1232 (1972).

HELLERMAN, L., CHUANG, H. Y. K., DE LUCA, D. C.: Approaches to the catalytic mechanism of mitochondrial monoamine oxidase. Adv. biochem. Psychopharmacol. **5**, 327–337.

HENZE, M.: *p*-Oxyphenyläthylamin, das Speicheldrüsengift der Cephalopoden. Hoppe-Seylers Z. physiol. Chem. **87**, 51–58 (1913).

HIDAKA, H., HARTMAN, B., UDENFRIEND, S.: Comparison of mitochondrial monoamine oxidases from bovine brain and liver using antibody to purified liver monoamine oxidase. Arch. Biochem. **147**, 805–809 (1971).

HILL, C. H., KIM, C. S.: The derangement of elastin synthesis in pyridoxine deficiency. Biochem. biophys. Res. Commun. **27**, 94–99 (1967).

HILL, J. M., MANN, P. J. G.: The inhibition of pea-seedling diamine oxidase by chelating agents. Biochem. J. **85**, 198–207 (1962).

HILL, J. M., MANN, P. J. G.: Further properties of the diamine oxidase of pea seedlings. Biochem. J. **91**, 171–182 (1964).

HÖKFELT, T.: Electron microscopic observations on nerve terminals in the intrinsic muscles of the albino rat iris. Acta physiol. scand. **67**, 255–256 (1966).

HÖKFELT, T., JONSSON, G.: Studies on reaction and binding of monoamines after fixation and processing for electron microscopy with special reference to fixation with potassium permanganate. Histochemie **16**, 45–67 (1966).

HOLTZ, P., HEISE, R., LÜDTKE, K.: Fermentativer Abbau von 1-Dioxyphenylalanin (Dopa) durch Niere. Naunyn-Schmiedebergs Arch. Pharmak. exp. Path. **191**, 87–118 (1938).

HOLTZ, P., PALM, D.: Brenzkatechinamine und andere sympathicomimetische Amine. Ergebn. Physiol. **58**, 1–580 (1966).

HOLTZ, P., STOCK, K., WESTERMANN, E.: Pharmakologie des Tetrahydropapaverolins und seine Entstehung aus Dopamin. Naunyn-Schmiedeberg's Arch. Pharmak. exp. Path. **248**, 387–405 (1964).

HOPE, D. B.: The metabolism of taurine and related compounds. D. Phil. Thesis, Oxford 1956.

HOPE, D. B., SMITH, A. D.: Distribution and activity of monoamine oxidase in mouse tissues. Biochem. J. **74**, 101–107 (1960).

HORITA, A., LOWE, M. C.: On the extraneuronal nature of cardiac monoamine oxidase in the rat. Advances in biochem. Psychopharmacol. **5**, 227–242 (1972).

HOUSLAY, M. D., TIPTON, K. F.: The nature of the electrophoretically separable multiple forms of rat liver monoamine oxidase. Biochem. J. **135**, 173–186 (1973).

HO-VAN-HAP, A., BABINEAU, L. M., BERLINGUET, L.: Hormonal action on monoamine oxidase activity in rats. Canad. J. Biochem. **45**, 355–362 (1967).

HUIKURI, K. T.: Histochemistry of the ciliary ganglion of the rat and the effect of pre- and postganglionic nerve division. Acta physiol. scand. **69**, suppl. **286**, 1–83 (1966).

HUNTER, N. W.: Intracellular localization of some hydrolases, and iron and copper porphyric enzymes in *Opalina carolinensis*. Trans. Amer. Microscop. Soc. **76**, 36–45 (1957).

HUNTER, N. W.: Enzyme patterns in *Paramecium putrinum* Claparède and Lachmann. Trans. Amer. Microscop. Soc. **78**, 363–370 (1959a).

HUNTER, N. W.: Enzyme systems of *Stylonychia pustulata* II. Miscellaneous systems (hydrases, hydrolases and dehydrogenases). Protozool. **6**, 100–104 (1959b).

HUNTER, N. W.: Enzyme systems of *Colpoda cucullus*. II. Intracellular activity of some enzymes as determined by histochemistry. Trans. Amer. Microscop. Soc. **80**, 38–43 (1961).

IGAUE, I., GOMES, B., YASUNOBU, K. T.: Beef mitochondrial monoamine oxidase, a flavin dinucleotide enzyme. Biochem. biophys. Res. Commun. **29**, 562–570 (1967).

IVERSEN, L. L.: The uptake and storage of noradrenaline in sympathetic nerves. Cambridge: University Press 1967.

IVERSEN, L. L.: Role of transmitter uptake mechanisms in synaptic neurotransmision. Brit. J. Pharmacol. **41**, 571–591 (1971).

IWATA, H., KARIYA, K.: Adrenergic mechanisms in *Tetrahymena*. I. Changes in monoamine oxidase activity during growth. Experientia (Basel) **29**, 265–266 (1973).

IWATA, H., KARIYA, K., OKAMOTO, H.: Amine oxidative system in *Tetrahymena pyriformis* W. Experientia (Basel) **27**, 388–390 (1971).

JANAKIDEVI, K., DEWEY, V. C., KIDDER, G. W.: The biosynthesis of catecholamines in two genera of protozoa. J. biol. Chem. **241**, 2576–2578 (1966).

JARROTT, B.: Occurrence and properties of monoamine oxidase in adrenergic neurons. J. Neurochem. **18**, 7–16 (1971).

JARROTT, B., IVERSEN, L. L.: Subcellular distribution of monoamine oxidase activity in rat liver and vas deferens. Biochem. Pharmacol. **17**, 1619–1625 (1968).

JARROTT, B., IVERSEN, L. L.: Noradrenaline metabolizing enzymes in normal and sympathetically denervated vas deferens. J. Neurochem. **18**, 1–6 (1971).

JOHNSTON, J. P.: Some observations upon a new inhibitor of monoamine oxidase in brain tissue. Biochem. Pharmacol. **17**, 1285–1297 (1968).

JONASON, J.: Metabolism of catecholamines in the central and peripheral nervous system. A study with special reference to the enzymes involved. Acta physiol. scand. Suppl. **320**, 1–50 (1969).

KAHLSON, G., ROSENGREN, E.: Biogenesis and physiology of histamine. Monograph No. 21 of the Physiol. Soc. London: Arnold 1971.

KAISER, W., BYGRAVE, F. L.: Incorporation of choline into the outer and inner membranes of isolated rat liver mitochondria. J. Biochem. **4**, 582–585 (1968).

KALSNER, S., NICKERSON, M.: A method for the study of mechanisms of drug disposition in smooth muscle. Canad. J. Physiol. Pharmacol. **46**, 719–730 (1968).

KALSNER, S., NICKERSON, M.: Mechanisms of cocaine potentiation of responses to amines. Brit. J. Pharmacol. **35**, 428–439 (1969a).

KALSNER, S., NICKERSON, M.: Effects of a haloalkylamine on responses to a disposition of sympathomimetic amines. Brit. J. Pharmacol. **35**, 440–455 (1969b).

KAPELLER-ADLER, R.: Amine oxidases and methods for their study, p. 1–319. New York-London-Sydney-Toronto: Wiley-Interscience 1970.

KAPELLER-ADLER, R., IGGO, B.: Histamine and its derivatives in human urine. Biochim. biophys. Acta (Amst.) **25**, 394–402 (1957).

KARLSSON, J.-O., SJÖSTRAND, J.: Synthesis, migration and turnover of protein in retinal ganglion cells. J. Neurochem. **18**, 749–767 (1971).

KEARNEY, E. B., SALACH, J. I., WALKER, W. H., SENG, R. L., KENNEY, W., ZESZOTEK, E., SINGER, T. P.: The covalently-bound flavin of hepatic monoamine oxidase. 1. Isolation and sequence of a flavin peptide and evidence for binding at the 8α position. Europ. J. Biochem. **24**, 321–327 (1971).

KIM, C. S., HILL, C. H.: The interrelationship of dietary copper and amine oxidase in the formation of elastin. Biochem. biophys. Res. Commun. **24**, 395–400 (1966).

KIM, H. C., D'IORIO, A.: Possible isoenzymes of monoamine oxidase in rat tissues. Canad. J. Biochem. **46**, 295–297 (1968).

KOELLE, G. B., VALK, A. DE T., JR.: Physiological implications of the histochemical localization of monoamine oxidases. J. Physiol. (Lond.) **126**, 434–447 (1954).

KOHN, H. I.: Tyramine oxidase. Biochem. J. **31**, 1693–1704 (1937).

KOPIN, I. J.: Storage and metabolism of catecholamines: The role of monoamine oxidase. Pharmacol. Rev. **16**, 179–191 (1964).

KREUTZBERG, G. W.: Neuronal dynamics and axonal flow IV. Blockage of intra-axonal enzyme transport by colchicine. Proc. nat. Acad. Sci. (Wash.) **62**, 722–728 (1969).

KUMAGAI, H., MATSUI, H., OGATA, K., YAMADA, H.: Properties of crystalline tyramine oxidase from *Sarcina lutea*. Biochim. biophys. Acta (Amst.) **171**, 1–8 (1969a).

KUMAGAI, H., MATSUI, H., OGATA, K., YAMADA, H., FUKAMI, H.: Oxidation of dopamine by crystalline tyramine oxidase from *Sarcina lutea*. Memoirs of the Research Institute for Food Science, Kyoto University, No. **29**, 69–71 (1968).

KUMAGAI, H., NAGATE, T., YAMADA, H., FUKAMI, H.: Characterization of sodium borohydride-reduced histaminase-histamine intermediate. Biochim. biophys. Acta (Amst.) **185**, 242–244 (1969b).

KUMAGAI, H., YAMADA, H., SUZUKI, H., OGURA, Y.: Action mechanism of tyramine oxidase from *Sarcina lutea*. J. Biochem. **69**, 137–144 (1971).

KUTTAN, R., RADHAKRISHNAN, A. N.: Studies on the biosynthesis of *sym*-homospermidine in sandal (*Santalum album L.*). Biochem. J. **127**, 61–67 (1972).

KVEDER, S., ISKRIC, S., KEGLEVIC, D.: 5-hydroxytryptophol: a metabolite of 5-hydroxytryptamine in rats. Biochem. J. **85**, 447–449 (1962).

LAKE, C. R., MILLS, R. R., BURNET, P. C. J.: β-hydroxylation of tyramine by cockroach hemolymph. Biochim. biophys. Acta (Amst.) **215**, 226–228 (1970).

LEHMANN, G., RANDALL, L. O.: Pharmacological properties of sympathomimetic diamines. J. Pharmacol. exp. Ther. **93**, 114–125 (1948).

LEMPINEN, M.: Extra-adrenal chromaffin tissue of the rat and the effect of cortical hormones on it. Acta physiol. scand. **62**, suppl. **231**, 1–91 (1964).

LENT, R., FRANZBLAU, C.: Studies on the reduction of bovine elastin: Evidence for the presence of $\Delta^{6,7}$-dehydrolysinonorleucine. Biochem. biophys. Res. Commun. **26**, 43–50 (1967).

LEVENE, C. I.: The lathyrogenic effect of isonicotonic acid hydrazide (INAH) on the chick embryo and its reversal by pyridoxal. J. exp. Med. **113**, 795–810 (1961).

LEVENE, C. I.: Studies on the mode of action of lathyrogenic compounds. J. exp. Med. **116**, 119–130 (1962).

LEVENE, C. I.: Effect of lathyrogenic compounds on the cross-linking of collagen and elastin *in vivo*. In: A Symposium on mechanisms of toxicity (ALDRIDGE, E., Ed.), p. 67–85. London: Macmillan 1971.

LEVENE, C. I., GROSS, J.: Alterations in state of molecular aggregation of collagen induced in chick embryos by β-aminopropionitrile (lathyrus factor). J. exp. Med. **110**, 771–790 (1959).

LIGHTMAN, S. L., IVERSEN, L. L.: The role of uptake 2 in the extraneuronal metabolism of catecholamines in the isolated rat heart. Brit. J. Pharmacol. **37**, 638–649 (1969).

LINDELL, S.-E., WESTLING, H.: Enzymic oxidation of some substances related to histamine. Acta physiol. scand. **39**, 370–384 (1957).

LINDSTRÖM, A., OLSSON, B., PETTERSSON, G.: Kinetics of the interaction between pig plasma benzylamine oxidase and substrate. Europ. J. Biochem. **35**, 70–77 (1973).

LINDSTRÖM, A., OLSSON, B., PETTERSSON, G.: Transient kinetics of benzaldehyde formation during the catalytic action of pig-plasma benzylamine oxidase. Europ. J. Biochem. **42**, 377–382 (1974).

LINDSTRÖM, A., PETTERSSON, G.: Active-site titration of pig plasma benzylamine oxidase with hydrazine derivatives. Europ. J. Biochem. **34**, 564–568 (1973).

LOVENBERG, W., DIXON, E., KEISER, H. R., SJOERDSMA, A.: A comparison of amine oxidase activity in human skin and rat liver: relevance to collagen cross-linking. Biochem. Pharmacol. **17**, 1117–1120 (1968).

MCCAULAY, R., RACKER, E.: Separation of two monoamine oxidases from bovine brain. Fed. Proc. **32**, 797 Abst. (1973).

MCEWEN, C. M.: Human plasma monoamine oxidase. I. Purification and identification. J. biol. Chem. **240**, 2003–2010 (1965).

MCEWEN, C. M.: The soluble monoamine oxidase of human plasma and sera. Advances in biochem. Psychopharmacol. **5**, 157–165 (1972).

MCLEAN, J. R., BURNSTOCK, G.: Axoplasmic flow of adrenaline and monoamine oxidase in amphibian sympathetic nerves. Z. Zellforsch. **124**, 44–56 (1972).

MANN, P. J. G.: Further purification and properties of the amine oxidase of pea seedlings. Biochem. J. **79**, 623–631 (1961).

MARTIN, G. R., PINNELL, S. R., SIEGAL, R. C., GOLDSTEIN, E. R.: Lysyl oxidase: The enzymatic step in collagen and elastin cross-linking. In: Chemistry and molecular biology of the intercellular matrix (BALAZS, E. A., Ed.), Vol. I, p. 405–410. London and New York: Academic Press 1970.

Medical Research Council: Biochemical research in psychiatry: survey and proposals. Report by a Council Committee, p. 11. London: Her Majesty's Stationery Office: 1970.

MONDOVI, B.: On the mechanism of amine oxidation by diamine oxidases. Advances in biochem. Psychopharmacol. **5**, 181–183 (1972).

MONDOVI, B., COSTA, M. T., FINAZZI-AGRO, A., ROTILIO, G.: Pyridoxal phosphate as a prosthetic group of pig kidney diamine oxidase. Arch. Biochem. **119**, 373–381 (1967a).

MONDOVI, B., ROTILIO, G., COSTA, M. T., FINAZZI-AGRO, A., CHIANCONE, E., HANSEN, R. E., BEINERT, H.: Diamine oxidase of pig kidney. Improved purification and properties. J. biol. Chem. **242**, 1160–1167 (1967b).

MONDOVI, B., ROTILIO, G., FINAZZI-AGRO, A., VALLOGINI, M. P., MALMSTRÖM, B. G., ANTONINI, E.: Copper reduction by substrate in diamine oxidase. FEBS Letters **2**, 182–184 (1969).

MÜLLER, E., PEARSE, A. G. E.: Localization of monoamine oxidase in mammalian and reptilian heart. Brit. Heart J. **27**, 116–120 (1965).

NAGATSU, T., NAKANO, G., MIZUTANI, K., HARADA, M.: Purification and properties of amine oxidases in brain and connective tissue (dental pulp). Advances in biochem. Psychopharmacol. **5**, 25–36 (1972).

NARAYANAN, A. S., SIEGEL, R. C., MARTIN, G. R.: On the inhibition of lysyl oxidase by β-aminopropionitrile. Biochem. biophys. Res. Commun. **46**, 745–751 (1972).

NEFF, N. H., GORIDIS, C.: Neuronal monoamine oxidase: specific enzyme types and their rates of formation. Advances in biochem. Psychopharmacol. **5**, 307–323 (1972).

NEUPERT, W., BRDICZKA, D., BÜCHER, TH.: Incorporation of amino acids into the outer and inner membrane of isolated rat liver mitochondria. Biochem. biophys. Res. Commun. **27**, 488–493 (1967).

NEUPERT, W., BRDICZKA, D., SEBALD, W.: Incorporation of amino acids in the outer and inner membrane of isolated rat liver mitochondria. In: Biochemical aspects of the biogenesis of mitochondrial (SLATER, E. C., TAGER, M., PAPA, S., QUAGLIARELLO, E., Eds.), p. 395–408. Bari: Adriatica 1968.

NIMMO-SMITH, R. H., RAISON, C. G.: Monoamine oxidase activity of *Schistosoma mansoni*. Comp. Biochem. Physiol. **24**, 403–416 (1968).

NIMNI, M. E., DESHMUKH, K., DESHMUKH, A.: Mechanism of inhibition of collagen cross-linking and depolymerization of an incompletely crosslinked form of insoluble collagen caused by D-penicillamine. In: Chemistry and Molecular Biology of the Intercellular Matrix (BALAZS, E. A., Ed.), p. 417–430. London-New York: Academic Press 1970.

NIMNI, M. E., DESHMUKH, K., GERTH, N.: Collagen defect induced by penicillamine. Nature New Biology **240**, 220–221 (1972).

NOVICK, W. J.: The effect of age and thyroid hormones on the monoamine oxidase of rat heart. Endocrinology **69**, 55–59 (1961).

O'DELL, B. L., HARDWICK, B. C., REYNOLDS, G., SAVAGE, J. E.: Connective tissue defect in the chick resulting from copper deficiency. Proc. Soc. exp. Biol. (N.Y.) **108**, 402–404 (1961).

OKAMOTO, H.: Influence of L-thyroxine on kynurenine 3-hydroxylase, monoamine oxidase, and rotenone-insensitive NADH-cytochrome C reductase in mitochondrial outer membrane. Biochem. biophys. Res. Commun. **43**, 827–833 (1971).

OKAMOTO, H., YAMAMOTO, S., NOZAKI, M., HAYAISHI, O.: On the submitochondrial localization of L-kynurenine-5-hydroxylase. Biochem. biophys. Res. Commun. **26**, 309–314 (1967).

OLIVECRONA, T., ORELAND, L.: Reassociation of soluble monoamine oxidase with lipid-depleted mitochondria in the presence of phospholipids. Biochemistry **10**, 332–340 (1971).

ORELAND, L.: Purification and properties of pig liver mitochondrial monoamine oxidase. Arch. Biochem. **146**, 410–421 (1971).

ORELAND, L.: Some properties of pig liver mitochondrial monoamine oxidase. Advances in biochem. Psychopharmacol. **5**, 37–43 (1972).

ORELAND, L., OLIVECRONA, T.: The role of acidic phospholipids in the binding of monoamine oxidase to the mitochondrial structure. Arch. Biochem. **142**, 710–714 (1971).

PAASONEN, M. K.: Inactivation of 5-hydroxytryptamine by mammalian blood platelets. Biochem. Pharmacol. **8**, 241–244 (1961).

PADMANABHAN, R., KIM, K. H.: Oxidation of spermidine by a *Pseudomonas*. Biochem. biophys. Res. Commun. **19**, 1–5 (1965).

PAGE, R. C., BENDITT, H. E. P.: Interaction of the lathyrogen beta-aminopropionitrile (BAPN) with a copper-containing amine oxidase. Proc. Soc. exp. Biol. (N.Y.) **124**, 454–459 (1967).

PAOLUCCI, F., CRONENBERGER, L., PLAN, R., PACHECO, H.: Purification et propriétés de la diamine: oxygène oxydo-reductase du placenta humain. Biochimie **53**, 735–749 (1971).

PARE, C. M. B.: Clinical implications of monoamine oxidase inhibition. Advances in biochem. Psychopharmacol. **5**, 441–444 (1972).

PARTRIDGE, S. M.: The part played by oxidation in the synthesis and breakdown of connective tissue. J. Soc. Leather Trades Chemistry **49**, 41–52 (1965).

PARTRIDGE, S. M., ELSDEN, D. F., THOMAS, J.: Constitution of the cross-linkages in elastin. Nature (Lond.) **197**, 1297–1298 (1963).

PARTRIDGE, S. M., ELSDEN, D. F., THOMAS, J., DORFMAN, A., TELSER, A., HO, P.-L.: Biosynthesis of the desmosine and isodesmosine cross-bridges in elastin. Biochem. J. **93**, 30–33C (1964).

PARTRIDGE, S. M., ELSDEN, D. F., THOMAS, J., DORFMAN, A., TELSER, A., HO, P.-L.: Incorporation of labelled lysine into the desmosine cross-bridges in elastin. Nature (Lond.) **209**, 399–400 (1965).

PAU, R. N., ACHESON, R. M.: The identification of 3-hydroxy-4-O-β-D-glucosidobenzyl alcohol in the left colleterial gland of *Blaberus discoidalis*. Biochim. biophys. Acta (Amst.) **158**, 206–211 (1968).

PEDERSEN, P. L., SCHNAITMAN, C. A.: The oligomycin-sensitive adenosine diphosphate-adenosine triphosphate exchange in an inner membrane matrix fraction of rat liver mitochondria. J. biol. Chem. **244**, 5065–5073 (1969).

PEGG, A. E., WILLIAMS-ASHMAN, H. G.: Biosynthesis of putrescine in the prostate gland of the rat. Biochem. J. **108**, 533–539 (1968a).

PEGG, A. E., WILLIAMS-ASHMAN, H. G.: Stimulation of decarboxylation of S-adenosylmethionine by putrescine in mammalian tissues. Biochem. biophys. Res. Commun. **30**, 76–82 (1968b).

PIEZ, K. A.: Cross-linking of collagen and elastin. Ann. Rev. Biochem. **37**, 547–570 (1968).

PIEZ, K. A., MILLER, E. J., LANE, J. M., BUTLER, W. T.: Localization of chemical and structural features on the collagen molecule. In: Chemistry and Molecular Biology of the Intercellular Matrix (BALAZS, E. A., Ed.), Vol. I, p. 117–125. London-New York: Academic Press 1970.

PINNELL, S. R., MARTIN, G. R.: The cross-linking of collagen and elastin in enzymatic conversion of lysine in peptide linkage to α-aminoadipic-δ-semialdehyde (allysine) by an extract from bone. Proc. nat. Acad. Sci. (Wash.) **61**, 708–716 (1968).

PUGH, C. E. M., QUASTEL, J. H.: Oxidation of aliphatic amines by brain and other tissues. Biochem. J. **31**, 286–291 (1937a).

PUGH, C. E. M., QUASTEL, J. H.: Oxidation of amines by animal tissues. Biochem. J. **31**, 2306–2321 (1937b).

RACKER, E., PROCTOR, H.: Reconstitution of the outer mitochondrial membrane with monoamine oxidase. Biochem. biophys. Res. Commun. **39**, 1120–1125 (1970).

RAGLAND, J. B.: Multiplicity of mitochondrial monoamine oxidases. Biochem. biophys. Res. Commun. **31**, 203–208 (1968).

RAINA, A.: Studies on the determination of spermidine and spermine and their metabolism in the developing chick embryo. Acta physiol. scand. **60**, suppl. **218**, 1–81 (1963).

RANDALL, L. O.: Oxidation of phenethylamine derivatives by amine oxidase. J. Pharmacol. exp. Ther. **88**, 216–220 (1946).

RAUCH, N., RAUCH, R. J.: Isoenzymes of amine oxidase in human plasma and other tissues. Experientia (Basel) **29**, 215–217 (1973).

RAUTERBERG, J., FIETZEK, P., REXRODT, F., BECKER, V., STARK, N., KÜHN, K.: The amino acid sequence of the carboxyterminal nonhelical cross link region of the α1 chain of calf skin collagen. FEBS Letters **21**, 75–79 (1972).

RICHTER, D.: Adrenaline and amine oxidase. Biochem. J. **31**, 2022–2028 (1937).

RILEY, W. D., SNELL, E. E.: Histidine decarboxylase of *Lactobacillus* 30a. V. Origin of enzyme-bound pyruvate separation of non-identical subunits. Biochemistry **9**, 1485–1491 (1970).

ROBERTSON, J. D.: Cell membranes and the origin of mitochondria. In: Regional Neurochemistry (KETY, S. S., ELKES, J.), p. 497–534. Oxford-London-New York-Paris: Pergamon Press 1961.

ROBINSON, R. G., GERSHON, M. D.: Synthesis and uptake of 5-hydroxytryptamine by the myenteric plexus of the guinea pig ileum: a histochemical study. J. Pharmacol. exp. Ther. **178**, 311–324 (1971).

ROENSCH, L. F., SAVAGE, J. E., O'DELL, B. L.: Purification and characterization of tropoelastin from copper deficient chick aorta. Fed. Proc. **31**, 480 Abst. (1972).

ROJKIND, M., HAMABATA, A., GONZÁLEZ, E., RENDON, G.: Intermolecular cross-links in rat skin and rat tail collagen. In: Chemistry and Molecular Biology of the Intercellular Matrix (BALAZS, E. A., Ed.), Vol. I, p. 293–303. London and New York: Academic Press 1970.

ROSS, L. L., GERSHON, M. D.: Electron microscopic radioautographic and fluorescence localization of sites of 5-hydroxytryptamine (5-HT) uptake in the myenteric plexus of the guinea-pig ileum. J. Cell Biol. **55**, 220a (1972).

RUCKER, R. B., O'DELL, B. L.: Connective tissue amine oxidase. I. Purification of bovine aorta amine oxidase and its comparison with plasma amine oxidase. Biochim. biophys. Acta (Amst.) **235**, 32–43 (1971).

SAMPATH, S. S., CLARKE, D. E.: A specific increase in intraneuronal monoamine oxidase activity in the rat vas deferens following adrenalectomy. Life Sci. **11** (Part I), 1037, 1–104 (1972).

SANDBERG, L. B., HACKETT, T. N., CARNES, W. H.: The solubilization of an elastin-like protein from copper-deficient porcine aorta. Biochim. biophys. Acta (Amst.) **181**, 201–207 (1969).

SANDBERG, L. B., ZEIKUS, R. D., COLTRAIN, I. M.: Tropoelastin purification from copper-deficient swine: a simplified method. Biochim. biophys. Acta (Amst.) **236**, 542–545 (1971).

SANDLER, M.: Catecholamine synthesis and metabolism in man: clinical implications (with special reference to Parkinsonism). In: Handbook of Experimental Pharmacology, Vol. 33 (BLASCHKO, H., MUSCHOLL, E., Eds.), p. 845–899. Berlin-Heidelberg-New York: Springer 1972.

SANDLER, M., YOUDIM, M. B. H.: Multiple forms of monoamine oxidase: functional significance. Pharmacol. Rev. **24**, 331–348 (1972).

SCHAYER, R. W.: Catabolism of physiological quantities of histamine *in vivo*. Physiol. Rev. **39**, 116–126 (1959).

SCHMIEDEBERG, O.: Über das Verhältnis des Ammoniaks und der primären Monoaminbasen zur Harnstoffbildung im Thierkörper. Naunyn-Schmiedebergs Arch. Pharmak. exp. Path. **8**, 1–14 (1877).

SCHMUTZLER, W., HAHN, F., GOLDSCHMIDT, O., SESEKE, G.: Untersuchungen über Substanzen mit histaminaseliberierender Wirkung beim Meerschweinchen. Naunyn-Schmiedebergs Arch. Pharmak. exp. Path. **255**, 344–352 (1966a).

SCHMUTZLER, W., HAHN, F., SESEKE, G., BERNAUER, W.: Über die Herkunft der Plasmahistaminase im anaphylaktischen Schock des Meerschweinchens. Naunyn-Schmiedebergs Arch. Pharmak., exp. Path. **252**, 332–338 (1966b).

SCHMUTZLER, W., KNOP, J.: Untersuchungen zur Substrat- und Inhibitor-Spezifität der freisetzbaren Leberhistaminase. Biochem. Pharmacol. **17**, 1141–1150 (1968).

SCHNAITMAN, C., ERWIN, V. G., GREENAWALT, J. W.: The submitochondrial localization of monoamine oxidase. An enzymatic marker for the outer membrane of rat liver mitochondria. J. Cell Biol. **32**, 719–735 (1967).

SCHNAITMAN, C., GREENAWALT, J. W.: Enzymatic properties of the inner and outer membranes of rat liver mitochondria. J. Cell Biol. **38**, 158–175 (1968).

SCHULZ, H.: Histochemische Untersuchungen an den Pansenzotten eines Hausyaks, *Bos mutus* f. *grunniens* L. Zool. Anz. **170**, 306–310 (1963).

SELIGMAN, A. M., UENO, H., MORIZONO, Y., WASSERKRUG, H. L., KATZOFF, L., HANKER, J. S.: Electron microscopic demonstration of dehydrogenase activity with a new osmiophilic ditetrazolium salt (TC–NBT). J. Histochem. Cytochem. **15**, 1–13 (1967).

SHAMBOUGH, C. F.: Bull. ent. Soc. Amer. **15**, 210 (1969). Quoted after Whitehead, 1970.

SHARMA, N. N., BOURNE, G. H.: Histochemical studies on the distribution of monoamine oxidase, simple esterase and glucose-6-phosphatase in *Balantidium coli* from chimpanzees. Acta histochem. (Jena) **17**, 293–301 (1964).

SHARMAN, D. F.: The catabolism of catecholamines. Brit. med. Bull. **29**, 110–115 (1973).

SHASKAN, E. G., SNYDER, S. H.: Kinetics of serotonin accumulation into slices from rat brain: relationship to catecholamine uptake. J. Pharmacol. exp. Ther. **175**, 404–418 (1970).

SHIELDS, G. S., COULSON, W. F., KIMBELL, D. A., CARNES, W. H., CARTWRIGHT, G. E., WINTROBE, M. W.: Studies in copper metabolism. XXXII. Cardiovascular lesions in copper-deficient swine. Amer. J. Path. **41**, 603–618 (1962).

SHIH, J.-H. C., EIDUSON, S.: Multiple forms of monoamine oxidase in developing brain: tissue and substrate specificities. J. Neurochem. **18**, 1221–1228 (1971).

SHIH, J. C., EIDUSON, S.: Monoamine oxidase (EC. 1.4.3.4): isolation and characterization of multiple forms of the brain enzyme. J. Neurochem. **21**, 41–49 (1973).

SILBERSTEIN, S. D., SHEIN, H. M., BERV, K. R.: Catechol-O-methyl transferase and monoamine oxidase activity in cultured rodent astrocytoma cells. Brain Res. **41**, 245–248 (1972).

SILVERMAN, M., EVANS, E. A.: The effects of spermine, spermidine and other polyamines on the growth inhibition of *Escherichia coli* by atabrine. J. biol. Chem. **154**, 521–534 (1944).

SIMPSON, G. G.: The principles of classification of mammals. Bull. Amer. Mus. Nat. Hist. (N.Y.) **85**, 1–350 (1945).

SMITH, C. L.: Stability of amphibian monoamine oxidase and the inhibitory action of semicarbazide. Comp. Biochem. Physiol. **1**, 305–318 (1960).

SMITH, D. W., BROWN, D. M., CARNES, W. H.: Preparation and properties of salt-soluble elastin. J. biol. Chem. **247**, 2427–2432 (1972).

SMITH, D. W., WEISSMAN, N., CARNES, W. H.: Cardiovascular studies on copper deficient swine. XII. Partial purification of a soluble protein resembling elastin. Biochem. biophys. Res. Commun. **31**, 309–315 (1968).

SMITH, T. A.: Polyamine oxidase in higher plants. Biochem. biophys. Res. Commun. **41**, 1452–1456 (1970).

SMITH, T. A.: The occurrence, metabolism and functions of amines in plants. Biol. Rev. **46**, 201–241 (1971).

SMITH, T. E., WEISSBACH, H., UDENFRIEND, S.: Studies on the mechanism of action of monoamine oxidase: metabolism of N,N-dimethyltryptamine and N,N-dimethyltryptamine-N-oxide. Biochemistry **1**, 139–143 (1962).

SNYDER, S. H., FISCHER, J., AXELROD, J.: Evidence for the presence of monoamine oxidase in sympathetic nerve endings. Biochem. Pharmacol. **14**, 363–365 (1965).

SOTTOCASA, G. L.: Biochemical properties of inner and outer mitochondrial membranes of liver. Biochem. J. **105**, 1P–2P (1967).

SOTTOCASA, G. L., KUYLENSTIERNA, B., ERNSTER, L., BERGSTRAND, A.: Separation and some enzymatic properties of the inner and outer membranes of rat liver mitochondria. In: Methods in Enzymology (COLOWICK, S. P., KAPLAN, N. O., Eds.), Vol. 10, p. 448–463. New York and London: Academic Press 1967.

SOURKES, T. L.: Properties of the monoamine oxidase of rat liver mitochondria. Advances in Pharmacol. **6A**, 61–69 (1968).

SOUTHGATE, J.: Endometrial monoamine oxidase: the effect of sex steroids. Advances in biochem. Psychopharmacol. **5**, 263–269 (1972).

SPINKS, A., BURN, J. H.: Thyroid activity and amine oxidase in liver. Brit. J. Pharmacol. 7, 93–98 (1962).

SQUIRES, R. F.: Multiple forms of monoamine oxidase in intact mitochondria as characterized by selective inhibitors and thermal stability: a comparison of eight mammalian species. Advances in biochem. Psychopharmacol. **5**, 355–370 (1972).

STARCHER, B. C.: The effect of pyridoxine deficiency on aortic elastin biosynthesis. Proc. Soc. exp. Biol. (N.Y.) **132**, 379–385 (1969).

STARCHER, B. C., PARTRIDGE, S. M., ELSDEN, D. F.: Isolation and partial characterization of a new amino-acid from reduced elastin. Biochemistry **6**, 2425–2432 (1967).

STARK, M., RAUTERBERG, J., KÜHN, K.: Evidence for a non-helical region at the carboxyl terminus of the collagen molecule. FEBS Letters **13**, 101–104 (1971).

STESINA, L. N., AKOPYAN, ZH. I., GORKIN, V. Z.: Modification of catalytic properties of amine oxidases. FEBS Letters **16**, 349–351 (1971).

STRÖMBLAD, B. C. R.: Supersensitivity and amine oxidase activity in denervated salivary glands. Acta physiol. scand. **36**, 137–153 (1956).

STRÖMBLAD, B. C. R.: Observations on amine oxidase in human salivary glands. J. Physiol. (Lond.) **147**, 639–643 (1959).

SYKES, B. C., PARTRIDGE, S. M.: Isolation of a soluble elastin from lathyritic chicks. Biochem. J. **130**, 1171–1172 (1972).

TABOR, C. W., KELLOGG, P. D.: Identification of flavin adenine dinucleotide and heme in a homogeneous spermidine dehydrogenase from *Serratia marcescens*. J. biol. Chem. **245**, 5424–5433 (1970).

TABOR, C. W., TABOR, H., BACHRACH, U.: Identification of the aminoaldehydes produced by oxidation of spermine and spermidine with purified plasma amine oxidase. J. biol. Chem. **239**, 2194–2203 (1964).

TABOR, H., TABOR, C. W.: Spermidine, spermine and related amines. Pharmacol. Rev. **16**, 245–300 (1964).

TANZER, M. L.: Isolation and partial characterization of intermolecule crosslinks from reconstituted collagen fibres. In: Chemistry and Molecular Biology of the Intercellular Matrix (BALAZS, E. A., Ed.), Vol. I, p. 287–292. London-New York: Academic Press 1970.

TANZER, M. L., HOUSLEY, T., BERUBA, L., FAIRWEATHER, R., FRANZBLAU, C., GALLOP, P. M.: Structure of two histamine containing crosslinks from collagen. J. biol. Chem. **248**, 393–402 (1973).

TANZER, M. L., KEFALIDES, N. A.: Collagen crosslinks: Occurrence in basement membrane collagens. Biochem. biophys. Res. Commun. **51**, 775–780 (1973).

TAYLOR, C. E., TAYLOR, R. S., RASMUSSEN, C., KNOWLES, P. F.: A catalytic mechanism for the enzyme benzylamine oxidase of pig plasma. Biochem. J. **130**, 713–728 (1972).

THEISS, E., SCHÄRER, K.: Toxicity of L-dopa and a decarboxylase inhibitor in animal experiments. In: Monoamines, noyaux gris centraux et syndrome de Parkinson (J. DE AJURIAGUERRA, G. GAUTHIER, Eds.), p. 497–504. Geneva: Georg; Paris: Masson 1971.

TIPTON, K. F.: The purification of pig brain mitochondrial monoamine oxidase. Europ. J. Biochem. **4**, 103–107 (1968a).

TIPTON, K. F.: The prosthetic groups of pig brain mitochondrial monoamine oxidase. Biochim. biophys. Acta (Amst.) **159**, 451–459 (1968b).

TIPTON, K. F.: Some properties of monoamine oxidase. Advances in biochem. Psychopharmacol. **5**, 11–24 (1972).

TIPTON, K. F.: Biochemical aspects of monoamine oxidase. Brit. Med. Bull. **29**, 116–119 (1973).

TIPTON, K. F.: Monoamine oxidase. In: Handbook of Physiology and Endocrinology (ROY, O. GREEP, E. B., ASTWOOD, Eds.). Washington: Amer. Physiol. Soc., sect. 7. In print.

TJÄLVE, H.: Catechol- and indolamines in some endocrine cell systems. An autoradiographical, histochemical and radioimmunological study. Acta physiol. scand. Suppl. **360**, 1–22 (1971).

TRANZER, J. P., THOENEN, H.: Electronmicroscopic localization of 5-hydroxytryptamine (3,4,5-trihydroxy-phenyl-ethylamine), a new "false" sympathetic transmitter. Experientia (Basel) **23**, 743–745 (1967).

TRAUB, W., PIEZ, K. A.: Chemistry and structure of collagen. Advanc. Protein Chem. **25**, 243–352 (1971).

TRENDELENBURG, U.: Thyroid and hyperglycaemia produced by adrenaline and noradrenaline. Brit. J. Pharmacol. **8**, 454–460 (1953).

TRENDELENBURG, U.: Factors influencing the concentration of catecholamines at the receptors. In: Handbook of Experimental Pharmacology, Vol. 33 (BLASCHKO, H., MUSCHOLL, E., Eds.), p. 726–761. Berlin-Heidelberg-New York: Springer 1972.

TRENDELENBURG, U., DRASKOCZY, P. R., GRAEFE, K. H.: The influence of intraneuronal monoamine oxidase on neuronal net uptake of noradrenaline and on sensitivity to noradrenaline. Advances in biochem. Psychopharmacol. **5**, 371–377 (1972).

UDENFRIEND, S.: Metabolism of 5-hydroxytryptamine. In: 5-Hydroxytryptamine (LEWIS, G. P., Ed.), p. 43–49. London-New York-Paris-Los Angeles: Pergamon Press 1958.

UTTENTHAL, L. O., HOPE, D. B.: Neurophysins and posterior pituitary hormones in the suiformes. Proc. roy. Soc. B **182**, 73–87 (1972).

VANE, J. R.: The relative activities of some tryptamine analogues on the isolated rat stomach preparation. Brit. J. Pharmacol. **14**, 87–98 (1959).

WALKER, W. H., KEARNEY, E. B., SENG, R. L., SINGER, T. P.: The covalently-bound flavin of hepatic monoamine oxidase. 2. Identification and properties of cysteinyl riboflavin. Europ. J. Biochem. **24**, 328–331 (1971).

WATANABE, K., SMITH, R. A., INAMASU, M., YASUNOBU, K. T.: Recent investigations on the prosthetic group of beef plasma amine oxidase. Advances in biochem. Psychopharmacol. **5**, 107–117 (1972).

WEAVER, R. H., HERBST, E. J.: The oxidation of polyamines by *Neisseria perflava*. J. biol. Chem. **231**, 647–655 (1958).

WEISSBACH, H., REDFIELD, B. G., UDENFRIEND, S.: Soluble monoamine oxidase; its properties and actions on serotonin. J. biol. Chem. **229**, 953–963 (1957).

WEISSMAN, N., SHIELDS, G. S., CARNES, W. H.: Cardiovascular studies on copper-deficient swine IV. Content and solubility of the aortic elastin, collagen and hexosamine. J. biol. Chem. **238**, 3115–3118 (1963).

WERLE, E., VON PECHMANN, E.: Über die Diamin-oxydase der Pflanzen und ihre adaptive Bildung durch Bakterien. Liebigs Ann. Chem. **562**, 44–60 (1949).

WERLE, E., TRAUTSCHOLD, I., AURES, D.: Reinigung und Charakterisierung der Diamin-Oxydase aus Erbsen Hoppe-Seyl. Z. physiol. Chem. **326**, 200–211 (1961).

WHITEHEAD, D. L.: The role of haemocytes in the biosynthesis of protocatechuate in the cockroach collaterial system. Biochem. J. **119**, 65–66P (1970).

WOHLRAB, F.: Der histochemische Nachweis der Monoaminoxydase mit thiazolyl-substituierten Tetrazoliumsalzen in Gegenwart von Metallionen. Histochemie **2**, 163–175 (1961).

YAGI, K., OZAWA, T., NAOI, M.: Mechanism of enzyme action. V. Demonstration of an initial step of enzyme-substrate complex using D-amino-acid oxidase and D-lactate or D-mandelate. Biochim. biophys. Acta (Amst.) **185**, 31–38 (1969).

YAMADA, H., ADACHI, O., OGATA, K.: Amine oxidases of microorganisms. Part II. Purification and crystallization of amine oxidase of *Aspergillus niger*. Agricult. biol. Chem. (Tokyo) **29**, 649–654 (1965a).

YAMADA, H., ADACHI, O., OGATA, K.: Amine oxidases of microorganisms. Part III. Properties of amine oxidase of *Aspergillus niger*. Agricult. biol. Chem. (Tokyo) **29**, 864–869 (1965b)

YAMADA, H., ADACHI, O., OGATA, K.: Amine oxidases in microorganisms. Part IV. Further properties of amine oxidase of *Aspergillus niger*. Agricult. biol. Chem. (Tokyo) **29**, 912–917 (1965c).

YAMADA, H., ADACHI, O., OGATA, K.: Putrescine oxidase, a diamine oxidase requiring flavin adenine dinucleotide. Agricult. biol. Chem. (Tokyo) **29**, 1148–1149 (1965d).

YAMADA, H., KUMAGAI, H., KAWASAKI, H., MATSUI, H., OGATA, K.: Crystallization and properties of diamine oxidase from pig kidney. Biochem. biophys. Res. Commun. **29**, 723–727 (1967a).

YAMADA, H., KUMAGAI, H., UWAJIMA, T., OGATA, K.: Bacterial monoamine oxidases. Part II. Substrate and inhibitor specificities of tyramine oxidase of *Sarcina lutea*. Agricult. biol. Chem. (Tokyo) **31**, 897–901 (1967b).

YAMADA, H., SUZUKI, H., OGURA, Y.: Amine oxidases from microorganisms: Stoichiometry of the reaction catalyzed by amine oxidase of *Aspergillus niger*. Advances in biochem. Psychopharmacol. **5**, 185–201 (1972).

YAMADA, H., TANAKA, A., OGATA, K.: Putrescine oxidase in *Micrococcus rubens*. Agricult. biol. Chem. (Tokyo) **29**, 260–261 (1965e).

YAMADA, H., UWAJIMA, T., KUMAGAI, H., WATANABE, M., OGATA, K.: Crystalline tyramine oxidase from *Sarcina lutea*. Biochem. biophys. Res. Commun. **27**, 350–355 (1967c).

YAMADA, H., UWAJIMA, T., KUMAGAI, H., WATANABE, M., OGATA, K.: Bacterial monoamine oxidases. Part I. Purification and crystallization of tyramine oxidase of *Sarcina lutea*. Agricult. biol. Chem. (Tokyo) **31**, 890–896 (1967d).

YAMADA, H., YASUNOBU, K. T.: Monoamine oxidase. I. Purification, crystallization and properties of plasma monoamine oxidase. J. biol. Chem. **237**, 1511–1516 (1962a).

YAMADA, H., YASUNOBU, K. T.: Monoamine oxidase. II. Copper, one of the prosthetic groups of plasma monoamine oxidase. J. biol. Chem. **237**, 3077–3082 (1962b).

YAMASAKI, E., SWINDELL, R., REED, D. J.: Some aspects of catalysis by the amine oxidase of pea seedlings. Biochemistry **9**, 1206–1210 (1970).

YASUNOBU, K. T., IGAUE, I., GOMES, L. B.: The purification and properties of beef liver mitochondrial monoamine oxidase. Advances in Pharmacol. **6A**, 43–59 (1968).

YASUNOBU, K. T., OI, S.: Mechanistic aspects of the bovine hepatic monoamine oxidase reaction. Advances in biochem. Psychopharmacol. **5**, 91–105 (1972).

YOUDIM, M. B. H.: Multiple forms of monoamine oxidase and their properties. Advances in biochem. Psychopharmacol. **5**, 67–77 (1972).

YOUDIM, M. B. H., SOURKES, T. L.: The flavin prosthetic group of purified rat liver mitochondrial monoamine oxidase. Advances in biochem. Psychopharmacol. **5**, 45–53 (1972).

YOUNG, J. Z.: The life of the Vertebrates, Second Edition. Oxford: Clarendon Press 1962.

ZELLER, E. A.: Über den enzymatischen Abbau von Histamin und Diaminen. Helv. chim. Acta **21**, 881–890 (1938).

ZELLER, E. A.: Diamine oxidase: specificity and mechanism. Advances in biochem. Psychopharmacol. **5**, 167–180 (1972).

ZELLER, E. A.: Third International Catecholamine Symposium, Strasbourg 1973. In print.

ZELLER, E. A., BARSKY, J., BERMAN, E. R.: Amine oxidases XI. Inhibition of monoamine oxidase by 1-isonicotinyl-2-isopropylhydrazine. J. biol. Chem. **214**, 267–274 (1955).

ZILE, M. H.: Effect of thyroxine and related compounds in monoamine oxidase activity. Endocrinology **66**, 311–312 (1960).

ZILE, M., LARDY, H. A.: Monoamine oxidase activity in liver of thyroid-fed rats. Arch. Biochem. **82**, 411–421 (1959).

ZUBRZYCKI, Z., STAUDINGER, H. J.: Kinetik, intrazelluläre Lokalisation und Induzierbarkeit der Monoaminoxydase. Hoppe-Seylers Z. physiol. Chem. **348**, 639–644 (1967).

These references have been added in proof:

BARDSLEY, W.C., CRABBE, M.J.C., SCOTT, I.V.: The amine oxidases of hnman placenta and pregnancy plasma. Biochem. J. **139**, 169–181 (1974).

MURPHY, D.L.: Technical strategies for the study of catecholamines in man. In: Frontiers in Catecholamine Research (USDIN, E., SNYDER, S.H., Eds.), p. 1077–1082. New York, Toronto, Oxford, Sydney and Braunschweig: Pergamon Press Inc. 1973.

SHANNON, W.A., WASSERKRUG, H.L., SELIGMAN, A.M.: The ultrastructural localization of monoamine oxidase (MAO) with tryptamine and a new tetrazolium salt, 2-(2'-benzothiazolyl)-5-styryl-3-(4'-phthalhydrazydyl) tetrazolium chloride (BSPT). J. Histochem. Cytochem. **22**, 170–182 (1974).

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