Endogenous inhibitors of the respiratory chain

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Endogenous respiratory inhibitors are widely distributed. They include proteins, fatty acids and some of their derivatives, and cyclic compounds. They act at various sites on the respiratory chain. They may trigger the degradation of mitochondria both in maturation and involution as well as serve for defense against invaders.

The energy production of a cell, primarily determined by the respiratory chain, is dependent on the number and size of the mitochondria. Their number is the resultant of synthesis and degradation, both of which might be subject to regulation. Whereas the biogenesis of mitochondria has found much interst, their degradation has attracted little attention. One might expect degradation to predominate under conditions which lead to a great decrease of the respiratory capacity or to its disappearance. Such situations are met in maturation and involution processes, such as maturation of the red cell or of the eye lens, cornification of the skin, and involution of the lactating mammary gland or of the uterus.

Particularly rewarding has been the study of the transition of the reticulocyte to the erythrocyte. It led to the discovery of specific proteins inhibiting the respiratory chain [1,2]. Free fatty acids and some of their derivatives proved also to be widespread inhibitors of respiration [3,4]. Other inhibitors, found in plants and micro-organisms, are cyclic compounds, while a variety of the inhibitions observed so far are unidentified [5,6].

Inhibitory proteins of the red cell

The reticulocyte, an immature red blood cell, is characterised by the absence (in mammals) or loss of function (in other vertebrates) of the nucleus and a nearly complete lack of the endoplasmic reticulum. Consequently the syntheses of DNA, of RNA, in part of phospholipids and, hence, of organelles cannot proceed. On the other hand, the reticulocyte possesses intact mitochondria and ribosomes. These are degraded during its transition to the mature erythrocyte. The degradation takes place within 1–3 days. An inhibitory protein (RF) of the respiratory chain, found in the cytosol of rabbit reticulocytes, suppresses both the NADH oxidase and the succinate oxidase system of disrupted mitochondrial preparations [1,7]; the action is exerted nearly universally on respiratory systems of animals and plants but not of *Escherichia coli* [7]. The sites of action of RF in the respiratory chain are the iron-sulphur protein regions in the succinate-ubiquinone and in the NADH-ubiquinone oxidoreductase segments; they seem to be identical with the sites of action of thenoyltrifluoroacetone and rotenone respectively [7]. Since the inhibition appears only when the respiratory chain is in the oxidised state, it was concluded that RF acts on the oxidised species of the iron-sulphur proteins; the inhibition is irreversible and appears to be due to a stoichiometric reaction.

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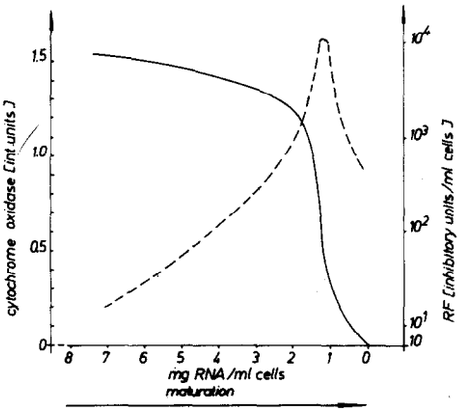

Fig. 1. Biological dynamics of mitochondrial constituents and the inhibitory protein RF during the maturation process of reticulocytes. The cytochrome oxidase (solid line), as a mitochondrial marker enzyme, and the RF activity (dotted line) are plotted in terms of the decreasing RNA content, as a measure of the age of the cell. The reticulocytes appearing in the course of an experimental bleeding anaemia were separated by density gradient centrifugation [11].

Some properties of RF are listed in Table I. RF is relatively stable only in crude extracts. Its inactivation is followed by an aggregation; divalent cations such as Mg^{2+}, Ca^{2+} and Ba^{2+}, protect the activity to a certain extent.

The activity of RF is strongly dependent on the type and degree of the maturation of the reticulocytes [7]. In the highly mature reticulocytes and erythrocytes of normal circulating blood, RF cannot be detected. In the very immature cells appearing at the 3rd and 4th day of strong daily bleeding little RF is present, but the 'megaloreticulocytes' poured out thereafter contain a large amount of RF. The dependence of the RF activity on the maturational stage of the cells is shown in Fig. 1. The maximal activity of RF appears in cells with a steep decline of mitochondrial constituents. From this behaviour it can be concluded that RF is intimately involved in the degradation of mitochondria and is one of the key factors which trigger the maturation of the reticulocytes.

In the reticulocyte supernatant a second inhibitory protein (RC) has been detected which acts on cytochrome oxidase [2,8]. How do the inhibitory proteins penetrate the two mitochondrial membranes? The probable answer was found recently in the detection of a further protein factor which exerts a lytic action on mitochondria in vitro and which has been identified as a lipoxygenase [9], the existence of which was assumed formerly to be restricted to plant materials. This enzyme causes holes to form in the membranes and so facilitates the penetration of the inhibitory proteins. In this manner, the lipoxygenase, RF, and RC accomplish in a synergistic way, with the aid of hydrolytic enzymes (proteinases and phospholipases), the complete degradation of mitochondria. This interplay is discussed in detail elsewhere [10].

TABLE I
Some molecular properties of the respiratory inhibitor protein of the reticulocyte (RF) [7]

Property	Evidence
Single polypeptide chain with MW of 80,000	SDS electrophoresis; ultracentrifugation
N-Terminal glycine	Dansylation method
Isoelectric point, pH 5.55	Isoelectric focusing
Carbohydrate moiety	Staining on electropherograms; anthrone method
Functional Fe^{2+}	Chelating agents
Essential SH group(s)	SH-reagents; amino-acid analysis
Affinity for phospholipids	Electrophoresis; inactivation by phospholipids
Tendency to aggregation and inactivation	Storage and disc-gel electrophoresis

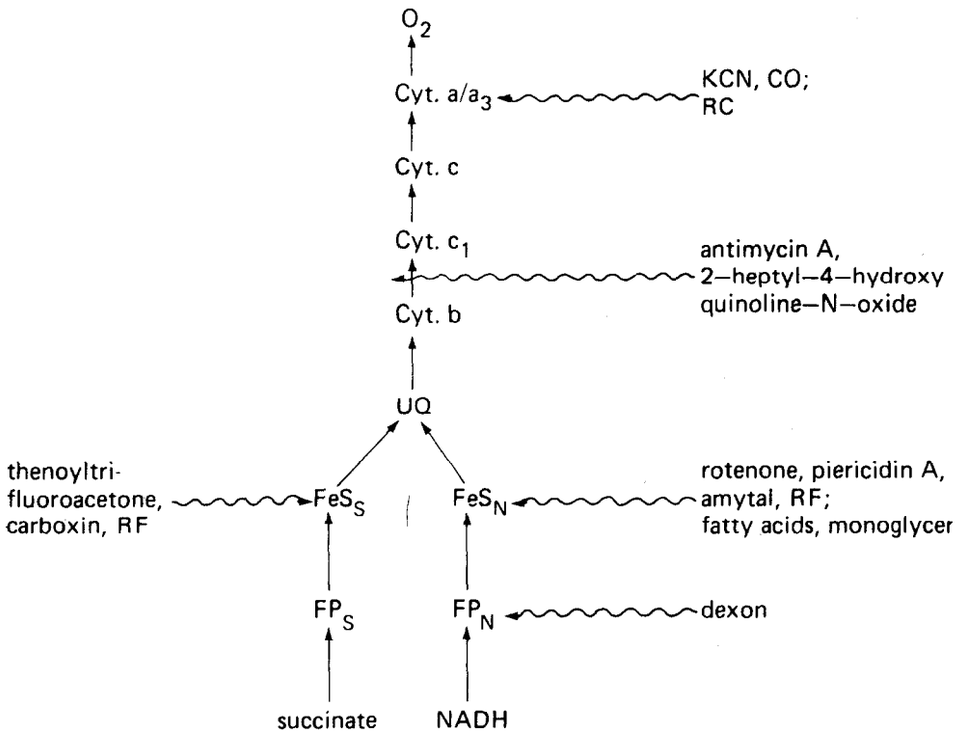

Fig. 2. The sites of action of some respiratory inhibitors. Straight arrows = paths of electron transfer; serpentine arrows = action of inhibitors; abbreviations: cyt. = cytochrome; UQ = ubiquinone, FP = flavoproteins, FeS = iron-sulphur proteins of the succinate oxidase (S) or the NADH oxidase (N) systems.

The degradation of mitochondria is a necessary event in the maturation of reticulocytes. It may yield a substantial biological benefit; the released amino acids are utilised as a source of energy and as building blocks for the synthesis of haemoglobin.

Free fatty acids as respiratory inhibitors

A ubiquitous class of endogenous respiratory inhibitors is represented by the free fatty acids and related compounds (CoA esters, monoglycerides and lysophosphatides). With respect to the mitochondrial metabolism the free fatty acids play a double role; they serve as substrates, but are also harmful agents. Normally the damaging effects may be prevented by their binding to intracellular proteins; the protection can be mimicked in vitro by serum albumin. A possible biological importance of the inhibitory effects of free fatty acids is suggested in cases where their intracellular level exceeds the binding capacity of the proteins for them. The increased cellular concentration of free fatty acids in hypoxia may be the cause of the injury of the mitochondrial respiratory system. A rise in free fatty acid content may also take place during developmental processes. The inhibitory effect of frog-egg homogenate on the respiratory chain, due to free fatty acids and monoglycerides, is strongly dependent on the developmental stage. Similarly the inhibitory effects of homogenates of rat testicles are related to their sexual maturity.

One may distinguish three kinds of inhibitory actions of free fatty acids on the respiratory chain. There is first an instant, completely reversible inhibition [3], secondly, a low irreversible effect, which is prevented but not relieved by serum albumin [4], and thirdly an irreversible thermally induced conformational change of the respiratory assembly [11]. All three effects are exerted preferentially on the iron-sulphur region of the NADH-ubiquinone oxidoreductase segment and are due to hydrophobic interactions rather than to non-specific detergent action.

An indirect inhibition of respiration by free fatty acids is also possible by way of activation of endogenous phospholipases. Furthermore, the fatty acids and their CoA esters uncouple oxidative phosphorylation, act as ionophores for alkali-metal ions, and inhibit the adenine nucleotide translocase.

TABLE II
Concentrations of half-inhibition of respiration exerted by respiratory inhibitors on electron-transfer particles from beef-heart mitochondria

Inhibitor	Concentration of half-inhibition*	
	[M]	[moles per mg protein]
Rotenone	5×10^{-9}	1×10^{-10}
RF	5×10^{-9}	1×10^{-10}
Antimycin A	5×10^{-8}	1×10^{-9}
2-Heptyl-4-hydroxyquinoline-*N*-oxide	5×10^{-7}	1×10^{-8}
Dexon	1×10^{-6}	2×10^{-8}
2-Hydroxy-3-alkylnaphtoquinone	3×10^{-6}	6×10^{-8}
Oleic acid-1-monoglyceride	4×10^{-6}	8×10^{-8}
Oleic acid	6×10^{-6}	1×10^{-7}
Lysolecithin (saturated)	1×10^{-5}	2×10^{-7}
Carboxin	2×10^{-5}	4×10^{-7}
KCN	2×10^{-5}	4×10^{-7}
Thenoyltrifluoroacetone	4×10^{-5}	8×10^{-7}
Amytal	8×10^{-4}	2×10^{-5}

* Rounded-off values.

Respiratory inhibitors in other animal cells

Inhibition of both the succinate oxidase and the NADH oxidase systems was detected with extracts from rat testicles, mammary gland, placenta, amnion, skeletal muscle, skin, and eye lens, while in several other tissue extracts from the rat, inhibition of the NADH oxidase system only was observed [5]. Further respiratory inhibitors in animal cells have been reported for example, in *Trypanosoma cruzi* [12], in *Tetrahymena pyriformis* [13], in nematocyst toxin of *Hydra littoralis* [14], in *Parazoanthus axinellae* [15], in sea urchin eggs [16], frog eggs [3], insect sarcosomes [17] polymorphonuclear leucocytes [18], and in acetone-treated beef-heart mitochondria [19]. Some of these inhibitions were identified as being due to free fatty acids, monoglycerides and lysophosphatides.

The inhibitor from *Parazoanthus axinellae*, which inhibits the succinate oxidase system of submitochondrial particles, was identified as a derivative of 1,3,5,7-tetrazacyclopent-[f]-azulene [15]. The inhibitor from leucocytes has turned out to be a mixture of low-molecular basic polypeptides which inhibit the cytochrome oxidase [18]. Even histones were shown to act as respiratory inhibitors [20]. The inhibitor of beef-heart mitochondria is a protein inhibiting the succinate-ubiquinone oxidoreductase system; it resembles the inhibitor RF from reticulocytes in being inactivated by phospholipids.

Low-molecular inhibitors in plants and micro-organisms

The respiratory inhibitor rotenone, which is also known as an insecticide and as a potent fish poison, occurs in the roots of some East-Asian Papilionaceae. Its action site seems to be an iron-sulphur protein component of the NADH-ubiquinone oxidoreductase complex [21]. Two other well-known low-molecular inhibitors, antimycin A and piericidin A, which have fungicidal and insecticidal actions, are of microbial origin. Antimycin interrupts the electron flux between cytochromes *b* and c_1, but the exact mechanism of action is not yet fully understood (see the review by Slater [22]). An antimycin-binding protein with a molecular weight of 11,500 was detected in the ubiquinone-cytochrome *c* oxidoreductase complex by photoaffinity labeling [23].

Another antibiotic, 2-heptyl-4-hydroxyquinoline-*N*-oxide acts in a similar fashion to antimycin A [24]; yet another antibiotic, acting like antimycin (funiculosin), was described recently [25]. Piericidin A seems to act like rotenone and at very low concentration; at higher concentrations it also inhibits the succinate oxidase activity by way of competition with ubiquinone [26].

Unidentified inhibitors in plants

No systematic studies on the distribution of endogenous respiratory inhibitors in plants and micro-organisms have been performed so far. From a pilot survey it is evident that inhibitors are widely distributed in the plant kingdom [6]. High inhibitory activities were observed in blossoms of *Forsythia intermedia*, male blossoms of *Corylus avellana*, inflorescences of *Brassiea oleracea L.* (cauliflower), fronds of *Pteridium aquilinum* and gall-nuts of *Quercus* leaves. The inhibitions of NADH oxidase were more numerous than of the succinate oxidase system. The specific inhibitory activities of these extracts (related to the dry mass) suggest concentrations of half-inhibition in the μM range or below. The inhibitory effects could not be accounted for by free fatty acids or their derivatives, but seemed to be due to low-molecular compounds in most cases. A further unidentified inhibitor of the NADH oxidase system was described in *Mucor* extracts [27].

Biological functions

Endogenous respiratory inhibitors are widely distributed in animals, plants and perhaps in micro-organisms. They are of different chemical natures (proteins, fatty acids and their derivatives, cyclic compounds) and have different sites of action in the respiratory chain (Fig. 2). The efficacies of the inhibitors differ by orders of magnitude (Table II). A common feature of nearly all inhibitors is a certain hydrophobicity. The hydrophobic character may be a prerequisite for the inhibitory action, since the components of the respiratory chain are located mainly in hydrophobic domains of the membrane.

The endogenous respiratory inhibitors may fulfil two biological functions. First, in maturation and involution processes they trigger the inactivation and degradation of mitochondria. In accordance with this function are the great variations seen in the inhibitor content of several animal and plant tissues, as well as reticulocytes, in different developmental stages. Secondly they may serve as a means of defense against foreign organisms. In this context it is of interest that some of the most useful fungicides (carboxin [28], dexon [29], tridemorph [30], pyrrolnitrin [31], and ethazol [32]) are inhibitors of the respiratory chain.

Other conceivable functions of the inhibitors in plants are to cause a switch-over to the alternative respiratory pathway existing in certain plants and fungi and induction and maintenance of a resting metabolism, for example, in dormancy. Thus, the study of respiratory inhibitors may prove to be rewarding both for basic and applied research.

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100 YEARS AGO

Hoppe-Seyler's Zeitschrift für Physiologische Chemie

The first issue of a scientific journal devoted only to biochemistry appeared in July 1877. Its name was 'Zeitschrift für Physiologische Chemie', the Editor-in-Chef Felix Hoppe-Seyler. After Hoppe-Seyler's death in 1895, the Journal was edited by Eugen Baumann [1] and Albrecht Kossel [2], and the name was changed to 'Hoppe-Seyler's Zeitschrift für Physiologische Chemie' in honour to its founder.

Around 1877, biochemistry was still in its infancy. From 1840 onwards, organic chemistry had developed rapidly; the language of structural formulae evolved (Kekule's benzene structure dates from 1865) and gave a firm basis to the chemistry of natural products, for example, alkaloids, amino acids, sugars. But the nature of proteins was still in the dark, nucleic acids had just been detected (Miescher, 1969), little was known about the enzymatic processes of digestion and virtually nothing about intermediary metabolism. It may have been for this reason that Miescher wrote to one of his friends (1874): 'Die physiologische Chemie besteht aus einem solchen Haufen unzusammenhängender Facta, daß es wenig Sinn hat, noch mehr Häckerling hinzutun zu wollen' [3].

But Hoppe-Seyler was more optimistic about contemporary biochemistry. Referring to the progress in organic chemistry, he wrote in the preface to the first issue of his journal:

In the last four decades, Organic Chemistry has encountered great advances. This enables us to investigate biological problems not only by analytical means but also to study the chemical processes of life experimentally. The admirable most recent results of chemical synthesis and the insights into the structure of chemical compounds as well as their structural changes by chemical reactions have provided us with means and ways to investigate with unexpected precision the ultimate causes of the processes of life, as far as structures and relations of the substances acting in the organism are concerned.

By this, biochemistry has grown up from its frist, naturally and necessarily analytical, beginnings to a science that not only places itself in the same ranks as biophysics but competes with the latter in activity and success; a glance into the annual reports of the achievements of these sciences suffices to place this fact beyond doubt. Though the great importance of physiological chemistry can hardly be questioned by anybody, this is apparently not realized by those most concerned with its scientific progress. Even today, physiological chemistry as a distinct science is taught either insufficiently or not at all in most German universities, and special lectures in this field are given only rarely.

It is evident from these lines that Hoppe-Seyler uses the terms 'biochemistry' and 'physiological chemistry' as synonyms. That he prefers 'physiological chemistry' in the title of the Journal may be related

Vorwort.

Der Aufschwung, welchen die organische Chemie in den letzten vier Jahrzehnten genommen hat, befähigt sie nicht allein die biologischen Probleme, wie es schon früher versucht war, in analytischer Richtung zu verfolgen, sondern auch experimentirend am lebenden Organismus die chemischen Lebensvorgänge eingehender Forschung zu unterwerfen. Die synthetischen Resultate, sowie die durch sie gewonnenen Einblicke in den Bau der chemischen Körper und seine Umgestaltungen durch chemische Processe, deren sich die jüngste Epoche rühmen darf, haben die Mittel und Wege gegeben, die Ursachen der Lebenserscheinungen in der Structur und Verwandtschaft der in den Organismen thätigen Stoffe mit einer früher nicht geahnten Sicherheit zu erforschen.

Die Biochemie ist hiefdurch aus ihren ersten natürlichen und nothwendigen analytischen Anfängen zu einer Wissenschaft erwachsen, welche nicht allein der Biophysik sich ebenbürtig an die Seite gestellt hat, sondern an Thätigkeit und Erfolgen ihr den Rang streitig macht; ein Blick in die Jahresberichte über die Leistungen dieser Wissenschaften genügt, um dies ausser Zweifel zu stellen. Obwohl nun kaum Jemand die hohe Bedeutung der physiologischen Chemie leugnen wird, ist sie doch offenbar noch zu wenig zum Bewusstsein derer gekommen, welche von dieser wissenschaftlichen Thätigkeit und ihren Fortschritten am nächsten berührt und in ihren eigenen Bestrebungen gefördert werden. Noch jetzt wird an den meisten deutschen Universitäten die physiologische Chemie als besondere Wissenschaft praktisch unzureichend oder gar nicht gelehrt und Vorträge über sie nur selten gehalten.

Part of the preface of the first issue of Hoppe-Seyler's Zeitschrift.