Monoamine Oxidase Activity in Membrane Structures of Rat Liver Cell

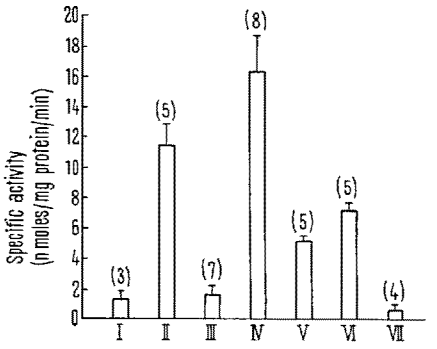
Monoamine oxidase activity in homogenates of liver, brain and some other organs is localized mainly in mitochondrial fraction[1] and is tightly bound with mitochondrial membranes[2]. On fractionation of mitochondrial membranes, the monoamine oxidase activity in some cases[3,4] was found in a fraction termed 'external mitochondrial membranes'. Monoamine oxidase (EC 1.4.3.4) has therefore been considered as a 'marker enzyme' for external mitochondrial membranes[4,5].

In homogenates of thyroid gland[6], heart musele[7] and of some other organs and tissues, a considerable part of monoamine oxidase activity has been localized in microsomes. However, direct evaluation, under comparable experimental conditions, of monoamine oxidase activity in various membrane structures of a cell has not been carried out.

It was one purpose of the present work to compare the values of specific monoamine oxidase activity (measured by a highly sensitive colorimetric method based on following the rate of oxidation of *p*-nitrophenylethylamine[8,9]) in various membrane structures of rat liver cell.

Preparations of mitochondrial membranes[10], cytoplasmic membranes[11], nuclei[12] and nuclear membranes (envelopes)[13], as well as those of membraneous structures of ergastoplasmic reticulum[14], were suspended in 0.2 *M* potassium phosphate buffer (pH 7.4). Content of protein has been measured as described by LOWRY et al.[15] using crystalline beef serum albumin as a standard.

The Figure shows that in mitochondrial membranes (which contain more than 70% of the total monoamine oxidase activity of rat liver homogenate[1]) specific monoamine oxidase activity calculated per mg of protein is considerably lower, as compared with the monoamine oxidase activity in cytoplasmic membranes or, especially, in nuclear membranes (envelopes). In some experimental hepatomas, this enzymatic activity is almost absent[16].



Specific monoamine oxidase activity in membrane structures of rat liver cell. Substrate *p*-nitrophenylethylamine. HCL[8]. Composition of samples and experimental conditions as described previously[9]. Mean values ∓ standard deviation are presented. Number of experiments in parentheses. I, starting homogenate; II, cytoplasmic membranes; III, nuclei; IV, nuclear membranes (envelops); V, mitochondria; VI, mitochondrial membranes; VII, membraneous structures of ergastoplasmic reticulum.

Monoamine oxidase activity may therefore not be considered as a characteristic property of mitochondrial membranes.

Biological significance of monoamine oxidase activity in membrane structures of cell is at the present time unknown. A possible role for these enzymes is suggested by the previously published data[17] on participation in regulation of activity of some structure – bound enzymes of tissue respiration of deaminated products of biogenic monoamines metabolism. Formation of these products is prevented by specific powerful monoamine oxidase inhibitors[17].

Вывод. Моноаминоксидазная активность присуща не только митохондриальным мембранам, но и некоторым другим мембранным структурам клетки.

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Enzymatic Phosphorylation of Proteins of Rat Liver Chromatin by (γ-^{32}P) ATP in vitro

The role played by chromosomal proteins in the control of gene activity is one of the main points of current interest. A characteristic of these proteins is the existence of several chemical groups on the amino acid side chains of the molecule, such as methyl, acetate and phosphate, groups which modify the net charge and perhaps the interaction of the protein with the nucleic acids of the chromatin[1]. It has been postulated and evidence has

been presented for a correlation between such chemical modifications of chromosomal proteins with increased rates of transcription[2].

We have shown previously[3] that isolated rat liver nuclei incorporate the γ-terminal phosphate of ATP into their proteins. Further we presented evidence that proteins of isolated chromatin are also phosphorylated in the presence of (γ-^{32}P) ATP. Some characteristics of the chromatin incorporating system are presented below.

Nucleotides were obtained from Boehringer, Mannheim, cyclic 3′,5′-AMP, Triton ×-100 and Dowex 50×4 from Serva, Heidelberg. $H_3{}^{32}PO_4$ (500–1000 Ci/mmole) and (γ-^{32}P) ATP (1–2 Ci/mmole) were purchased from The Radiochemical Centre, Amersham, $Na_4{}^{32}P_2O_7$ (10–70 mCi/mmole) from C.E.A., Gif-sur-Yvette. Male Wistar BRII rats weighing 120–170 g were used.

Isolation of nuclei and chromatin: Rat liver nuclei were isolated as described previously[4]. Starting with purified nuclei chromatin was then prepared according to MARUSHIGE and BONNER[5].

Separation of chromosomal proteins: The method adopted to separate chromosomal proteins is in principle the one used by GILMOUR and PAUL[6]. Chromatin from 10 rat livers was taken up in 80–100 ml 2*M* NaCl, 5*M* urea in a buffer containing 1 m*M* mercaptoethanol, 0.5 m*M* $MgCl_2$, 1 m*M* EDTA and 10 m*M* *Tris*-HCl pH 8.0. After stirring for 1 h the solution was centrifuged at 64,000*g* in a Beckmann L2 65 ultracentrifuge for 8 h. The pellet was once more extracted with the above solution of saline-urea. The proteins still remaining on the DNA were called residual proteins. The clear supernatant was dialyzed against 20 l of 5*M* urea, 1 m*M* mercaptoethanol, 0.5 m*M* $MgCl_2$, 1 m*M* EDTA, 5 m*M* *Tris*-HCl pH 8.0 for ca. 8 h. The dialyzed solution was run over a QAE-Sephadex A25 column, was washed until no further trichloroacetic acid (TCA) precipitable material could be recovered, and the acidic proteins were eluted by 2*M* NaCl, 5*M* urea, 1 m*M* mercaptoethanol. 0.5 m*M* $MgCl_2$, 1 m*M* EDTA, 5 m*M* *Tris*-HCl pH 8.0, The non-adsorbed basic proteins (histones) were precipitated by 10 volumes of cold acetone. The material eluted with 2*M* NaCl was designated as acidic proteins. During all preparations the temperature was kept below + 5 °C.

Identification of (^{32}P) phospho-serine and (^{32}P) phospho-threonine in hydrolysates of chromosomal proteins phosphorylated by (γ-^{32}P) ATP was performed according to SCHAFFER et al.[7].

Measurement of incorporated radioactivity: 2 methods were used to determine the uptake of label into nuclear proteins as described previously[3]. Protein was determined according to LOWRY et al.[8].

The time course of the uptake of the γ-terminal phosphate of ATP into chromosomal proteins is shown in Figure 1, together with the dependence on Mg^{++} (Figure 1a) and Mn^{++} (Figure 1b). The phosphorylation of the nuclear proteins is significantly stimulated by the presence of either Mg^{++} or Mn^{++} ions. With Mg^{++} concentrations above $10^{-2}M$, the incorporation rises in the first minutes of incubation and then decreases, due to the rapid depletion of substrate on the one hand by the ATPase present in the chromatin[3], and to the enzymatic dephosphorylation on the other hand. Thermal denaturation of chromatin (10 min at 100 °C) effectively reduces the incorporation of phosphate in chromosomal proteins, as well as the release of previously incorporated label.

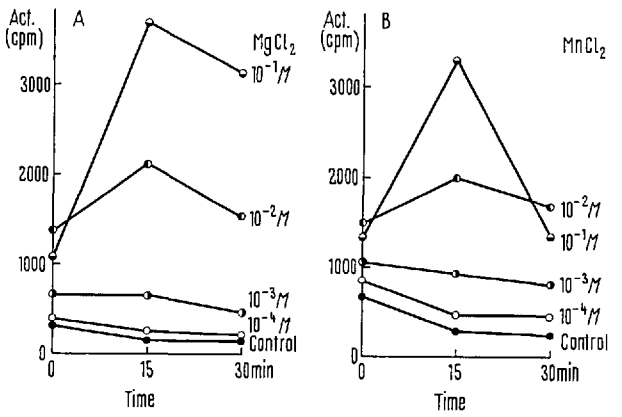

Fig. 1. Phosphorylation of chromatin in dependence of Mg^{++} and Mn^{++}. 0.2 ml of chromatin (400 µg protein) in 0.01*M* *Tris*-HCl pH 8.0 were mixed at 0 °C with 0.1 ml (γ-^{32}P) ATP (0.5 µCi) and 0.1 ml $MgCl_2$ resp. $MnCl_2$ in 0.01*M* *Tris*-HCl pH 8.0 to give the concentration indicated in the figures. The mixture was incubated in a water bath at 37 °C and 0.1 ml aliquots were taken at 0, 15 and 30 min to measure the radioactivity (see methods).

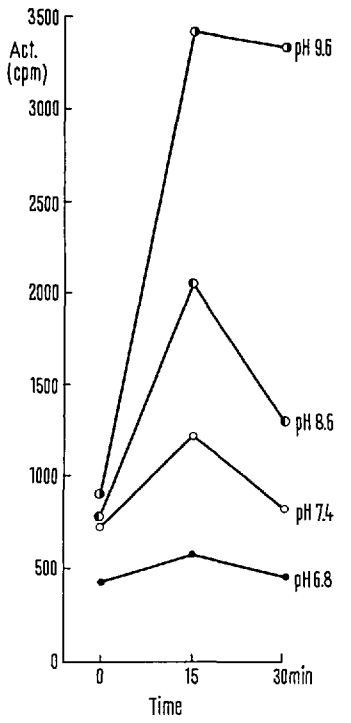

Fig. 2. Phosphorylation of chromatin in dependence of pH. 0.4 ml of chromatin (600 µg protein) in 0.01*M* *Tris*-HCl pH 8.0 were mixed at 0 °C with 0.1 ml (γ-^{32}P) ATP (0.5 µCi) in $5\times10^{-2}M$ $MgCl_2$, 0.01*M* *Tris*-HCl pH 8.0 and 0.1 ml of a 0.1*M* *Tris* buffer with a pH so as to give the wanted pH to the final solution. The mixture was incubated in a water bath at 37 °C and 0.1 ml aliquots were taken at 0, 15 and 30 min to measure the radioactivity (see methods).

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As evident from Figure 2, the phosphorylation is greatly favored by raising the pH of the incubation mixture. Since chromatin forms strong gels above pH 9–10, higher pH values were not tested. Phosphate and pyrophosphate enhance the incorporation (Table I) while, in the presence of $CuSO_4$, the phosphorylation is strongly inhibited. Monovalent cations show no influence. The effect of the presence of non-labelled nucleoside triphosphates and of cyclic 3′,5′-AMP is shown in Table I. $10^{-3}M$ ATP dilutes the labelled ATP and therefore leads to a decreased incorporation of the labelled phosphate. CTP, UTP and cyclic AMP in the same concentrations have also an inhibitory influence on the phosphorylation, whereas GTP shows no effect. Cyclic AMP at high concentrations inhibits, whereas at lower concentrations ($10^{-5}M$) has no significant influence on the phosphorylation of chromatin.

^{32}P-pyrophosphate is not incorporated to a measurable extent. ^{32}P-orthophosphate is adsorbed to chromosomal proteins. In hydrolysates of chromosomal proteins incubated with ^{32}P-*ortho*phosphate, the label was recovered as *ortho*phosphate, whereas in the case of phosphorylation by (γ-^{32}P) ATP it was recovered partly as phosphoserine and phosphothreonine (Figure 3).

The distribution of label between the different chromosomal proteins is shown in Table II. Separation was accomplished by first dissociating the bulk of the proteins with $2M$ NaCl and $5M$ urea as described in methods and then separating the proteins in histones and acidic proteins by QAE-Sephadex chromatography. About 3–5% of the proteins remain tightly bound to the DNA and cannot be released by usual procedures. (For further characterization of the residual nucleo-protein see [9]). Both histones and acidic proteins are phosphorylated. However, the phosphorylation of the acidic proteins is much higher than that of the histones. BENJAMIN and GOODMAN[10] have recently described a phosphorylation of rat liver nuclei and chromatin by ATP. They also showed that nonhistone proteins of giant salivary gland chromosomes of *Sciara coprophila* larvae are actively phosphorylated first in puffed regions, later all over the chromosomes. The phosphorylation of chromosomal proteins has been related to increased gene activity by several authors. It is still premature to ascribe a precise function to the chromosomal proteins or to their chemical modifications. In any case, since phosphorylating enzymes a well as methylating[11–13] and acetylating[14] enzymes are present in the chromatin, a direct stimulation of

Table I. Effects of phosphate, pyrophosphate, Cu^{++} and nucleotides

Agent	Incorporation (cpm)
Control	2083
$10^{-3}M$ phosphate	2427
$10^{-2}M$ phosphate	2846
$10^{-3}M$ pyrophosphate	2125
$10^{-2}M$ pyrophosphate	2282
$10^{-3}M$ Cu^{++}	982
Control	1840
$10^{-3}M$ ATP	617
$10^{-3}M$ GTP	1854
$10^{-3}M$ UTP	1517
$10^{-3}M$ CTP	1386
Control (cycl. AMP)	1871
$10^{-3}M$	1126
$10^{-5}M$	1830
$10^{-7}M$	2033
$10^{-9}M$	2000
$10^{-11}M$	1995

0.3 ml of chromatin (500 µg protein) in 0.01 M *Tris*-HCl pH 8.0 were added to 0.1 ml of $5\times10^{-2}M$ $MgCl_2$, 0.01 M *Tris*-HCl pH 8.0 containing 0.5 µCi (γ-^{32}P) ATP and 0.1 ml of the substance to be tested in 0.1 M *Tris*-HCl pH 8.0 at 0 °C. After 10 min of incubation for the phosphates and Cu^{++}, 12 min for the nucleotides and 8 min for cycl. 3′,5′-AMP in a water bath at 37 °C, 0.1 ml aliquots of the chromosomal suspension were taken for measurement of phosphorylation (see methods). The standard deviations were usually 5–10%.

Table II. Distribution of label in different chromosomal protein fractions

Preparation	Protein-bound activity (cpm/mg protein)
Dialyzed supernatant of the 65,000 g centrifugation	2640
Residual proteins	5043
Histones	470
Acidic proteins	11400

40 ml of chromatin from 10 rat livers (65 mg protein) in $10^{-2}M$ $MgCl_2$, 0.01 M *Tris*-HCl pH 8.0 containing 100 µCi (γ-^{32}P) ATP were incubated for 10 min at 37 °C. The reaction was stopped by adding 40 ml of a cold suspension of $4M$ NaCl, $10M$ urea, 0.01 M *Tris*-HCl pH 8.0. Proteins were separated as described in methods.

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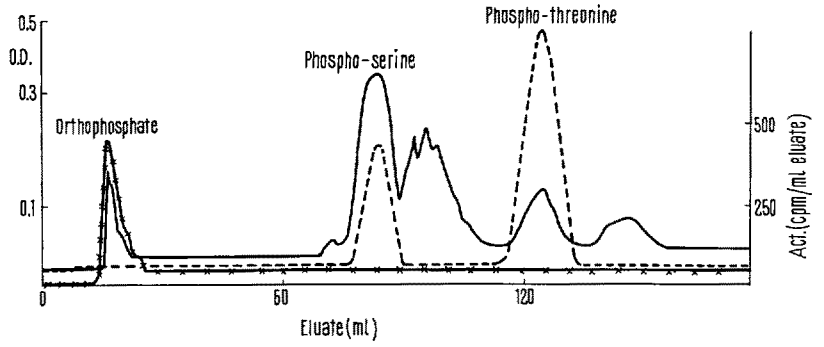

Fig. 3. Identification of (^{32}P) phospho-serine and (^{32}P) phosphothreonine in hydrolysates of chromosomal proteins phosphorylated by (γ-^{32}P)ATP (see methods). ---, marker-substances added before chromatography. —, hydrolysate of proteins phosphorylated by (γ-^{32}P)ATP. ×–×–×, hydrolysate of proteins incubated with (^{32}P) *ortho*-phosphate.

these enzyme systems by different agents acting on chromosomal functions could be visualized[15].

Zusammenfassung. Isoliertes Rattenleberchromatin baut die endständige Phosphat-Gruppe von (γ-^{32}P) ATP in seine Proteine in Form von Phosphatester von Serin und Threonin ein. Der Einbau ist Mg^{++} und Mn^{++} abhängig und steigt mit Erhöhung des pH des Inkubationsmediums an. Phosphat und Pyrophosphat stimulieren die Phosphorylierung, zyklisches AMP hat keinen Effekt. Die sauren Proteine werden viel stärker als die Histone phosphoryliert. Das eingebaute Phosphat wird sehr schnell freigesetzt, ein Prozess, der von der Anwesenheit von dephosphorylierenden Enzymen abhängt.

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Effect of Methotrexate on DNA Synthesis and Thymidine Kinase Activity of Human Lymphocytes Stimulated with Phytohaemagglutinin

The folic acid analogue Methotrexate[1] (4-amino-10-methyl pteroyl glutamic acid) is known to inhibit DNA synthesis mainly by blocking the conversion of deoxyuridylate to deoxythymidylate[2-5]. Also associated with the action of MTX are a number of changes in the activities of the enzymes of the DNA synthetic pathway. However, with the exception of the dihydrofolate reductase activity which is generally decreased a short time after MTX[4-7], the activities of other enzymes exhibit a wide range of variability depending on the experimental system employed. In cultures of Chang human liver cells Eker[8] has found an increase of thymidine kinase activity following the addition of MTX. On the contrary Labow, Maley and Maley[9] showed that in regenerating liver MTX completely inhibits the stimulation of deoxycytidylate deaminase, thymidine kinase and thymidylate kinase but increases the activity of thymidylate synthetase.

In PHA stimulated human lymphocytes MTX has been used as a synchronizing agent. It has been shown to reversibly block cells in S phase[10], but the biochemical events related to this block are still unclear.

In the present work we studied the effect of MTX on the incorporation of ^{3}H-thymidine into DNA of stimulated human lymphocytes. MTX induced an increase of incorporation of exogenous thymidine into DNA. For this reason in order to assay the real DNA synthetic activity, ^{32}P incorporation into DNA was evaluated. We also studied the effect of MTX on thymidine kinase activity to rule out the possibility that an increase of the thymidine phosphorylation could be related to the increased thymidine incorporation into DNA.

Methods and material. Human blood was drawn from healthy volunteers of both sexes, mixed with heparin (20 units/ml) and 4 volumes of blood, 1 volume of Plasma gel (Roger Bellon – France) and allowed to sediment at 37 °C for 1–2 h. The supernatant was passed through a nylon wool column (Filtralon) where 90–98% of the pagocytic cells were retained[11]. The lymphocytes were collected by centrifugation, resuspended (1.5×10^6 cells/m^3) in TC 199 containing 20% of autochthonous plasma and distributed in 7 ml aliquots into glass screw-cap flasks of 25 ml total capacity. PHA (Burroughs, Wellcome) was added to a concentration of 10 µl/ml of culture and Methotrexate (Lederle), unless otherwise stated, to a concentration of 5 µg/ml of culture. MTX, when added, was present from the initiation of the cultures. All the cultures were harvested 48 h after the addition of PHA.

In order to study DNA and RNA synthesis ^{3}H-TdR (5 Ci/m*M*, Radiochemical Centre – Amersham) 1 µc/ml and ^{32}P as H_3PO_4 (Sorin – Saluggia) 5 µc/ml were added 1 h and 3 h respectively before harvesting the cultures.

[1] The following abbreviations are used: MTX, methotrexate; PHA, phytohaemagglutinin; TdR, thymidine; dTTP, thymidine triphosphate.

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Table I. Effects of MTX on the percent of cells in S phase, on the incorporation of ^{3}H-TdR and ^{32}P into DNA and on the incorporation of ^{32}P into RNA

Treatment	Labelled cells (%)	^{3}H/µg of DNA (count/min)	^{32}P/µg of DNA (count/min)	^{32}P/µg of RNA (count/min)
Controls (PHA only)	30	1563 ± 438[a] (8)[b]	119.8 ± 16 (6)	1851 ± 285 (3)
Methotrexate (MTX and PHA)	37	5009 ± 1107 (6)	59.3 ± 7.5 (6)	1785 ± 74 (3)