was added and the yellow color of the *p*-nitrophenol released from the substrate by the enzyme was read at 400 nm. A unit of enzyme was defined as the amount required to hydrolize 1 µmole substrate per min. ε 400 of *p*-nitrophenol is 12,000[12].

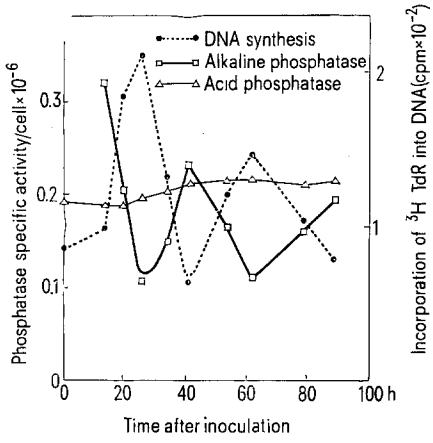
Acid phosphatase was assayed as previously described[13] except that the buffer in the reaction mixture was 0.1 *M* sodium acetate, pH 5.

Results and discussion. Alkaline phosphatase activity was highest when DNA synthesis was lowest and vice versa (Figure). Acid phosphatase activity, however, remained almost constant throughout several cell cycles as previously described[14–16].

The inverse relationship between DNA synthesis and alkaline phosphatase might indicate a way in which the enzyme is involved in DNA metabolism. If the natural substrate in the cell for alkaline phosphatase is inorganic pyrophosphate and this pyrophosphatase activity is needed to render nucleic acid biosynthesis irreversible by cleaving the pyrophosphates, which are by-products of nucleic acid synthesis[5–10], then its activity in the cell should be highest during the S-phase. Our results do not support this hypothesis. It is possible that alkaline phosphatase and pyrophosphatase, which are thought to be associated with a single enzyme[5–10], are actually separate entities. This idea has recently gained support from a work by HERZ and KAPLAN[16] which shows that in contrast to its striking effect on alkaline phosphatase activity in several cell lines, human serum does not modulate the activity of inorganic pyrophosphatase to a significant extent.

If alkaline phosphatase is involved as transphosphorylase in controlling the level of nucleotides in the cell as suggested[2,3], then its activity in the cell should be highest after completion of the S phase. At this stage the cell should start building up its free nucleotide pool, which is exhausted during DNA synthesis, in preparation for the next cycle of DNA syntheis. Our results support this hypothesis but do not exclude the possibility that alkaline phosphatase also has other biological duties in the cell.

Our results disagree with REGAN's study[17] which failed to find such relationship between alkaline phosphatsae and DNA synthesis in HeLA cells that were synchronized with 5-aminouracil.



Acid phosphatase and alkaline phosphatase activities in relation to DNA synthesis in synchronized HeLa S3 cells. 3 synchronized cultures were pulse labelled at various time intervals with ^{3}H TdR; cells were harvested, sonicated, and assayed in triplicate for acid and alkaline phosphatase and for acid-insoluble ^{3}H TdR incorporation into DNA. The detailed procedure is described in Materials and Methods. △, acid phosphatase; □, alkaline phosphatase; ●, acid-insoluble cpm ^{3}H TdR incorporated into DNA.

Zusammenfassung. In synchronisierten HeLa S3-Zellen wurde die Aktivität der alkalischen Phosphatase in Bezug auf den Zellteilungszyklus untersucht. Die geringste Aktivität wurde während der S-Phase und die höchste beim Minimum der DNS-Syntheseaktivität festgestellt, und es wird angenommen, dass die alkalische Phosphatase als Transphosphorylase zur Regulation der Polgrösse von Nucleotiden dient.

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Substratum Specificity of Purified Peroxidase Isoenzymes of Horse Radish Root

Peroxidase is an enzyme present in almost all higher plants[1], in human saliva[2], in the medular part of the suprarenal gland[3], in liver[4], kidneys[5], leukocytes[6]. The peroxidase isolated from horse radish root was much studied, being almost completely characterized[7–9]. This enzyme being highly specific for hydrogen peroxide, in whose presence it catalyses the oxidation of certain substances such as: phenols, aromatic amines, triptofan, bilirubin, pyrogalol, benzidine, etc.

The purpose of the present work consists in separating horse radish peroxidase isoenzymes by the chromatography on column method, and in analyzing the specificity of different peroxidase fractions for peroxidic groupings contained in the H_2O_2 ROOH, ROOR type compounds (in which R is the cumyl radical).

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Material and method. Enzyme extraction was performed from horse radish acetonic powder, at the temperature of 4 °C for 24 h, with a K_2HPO_4, 1×10^{-1} *M* solution (1 g/20 ml). The resulting homogenate was centrifuged at 12,000 rpm for 15 min and the supernatant was used for the peroxidase isoenzymes purification. For this purpose, total proteinic extract was absorbed on a Sephadex G-50 medium column (2,8 × 25 cm), equilibrated in a acetic acid-acetate buffer solution, 5×10^{-3} *M*, pH 4.4. The column elution was achieved with the same buffer solution, at a rate of 13 ml/h and 5 ml fractions were collected. The active eluates obtained on this column were reunited and passed on a CM-cellulose column (1.8 × 19 cm), equilibrated in an acetic acid-acetate buffer solution, 5×10^{-3} *M*, pH 4.4. Protein elution was effected by the successive introduction of the following solutions: 20 ml acetic acid-acetate buffer solution, 5×10^{-3} *M*, pH 4.4; 20 ml acetic acid-acetate buffer solution, 5×10^{-2} *M*, pH 4.4; 20 ml acetic acid-acetate buffer solution, 6×10^{-2} *M*, pH 4.4; 20 ml acetic acid-acetate buffer solution, 1×10^{-1} *M* and 40 ml acetic acid-acetate buffer solution, 25×10^{-2} *M*, pH 4.9. Elution rate was of 20 ml/h at 7 °C, 2 ml fractions being collected.

Eluted proteinic fractions from the Sephadex G-50 medium column were analysed by electrophoresis in agar gel 1,25% (250 V, 3 mA per plate, 4 migration h). Protein dosing was performed according to the method described by LOWRY et al.[10]. Enzymatic activity measurement was realized by BRAD'S et al.[11] method. Specific enzymatic activity was expressed in conformity with EC of I.U.B.: μmoles ascorbic acid/mg protein/minute/25 °C.

All the reagents used in the present work were furnished by the following concerns: Merck, BDH, Chemapol, Reactivul Bucureşti.

Results and discussions. The optimum total protein extraction conditions, in general, and of protein with peroxidasic activity, especially, were tested. Of the 3 extraction media: distilled water, acetic acid-acetate buffer solution, 5×10^{-3} *M*, pH 4.4 and K_2HPO_4 solution, 1×10^{-1} *M*; the latter proved the most efficient. By increasing the extraction time, it was recorded that in 24 h the greatest quantity of protein with peroxidasic activity is extracted.

Attempts to purify the enzyme by fractioned ammonium sulphate precipitations were unsuccessful, a purification factor of less than 2 being obtained.

By chromatography on a Sephadex G-50 medium column, an enzyme purification of 4.2; 3.2 and 5.5 times is achieved as against the total proteinic extract, if activity is determined in the presence of hydrogen peroxide, of cumene hydroxiperoxide and respectively of peroxide dicumene.

In Figure 1, the protein elution curve is shown on the CM-cellulose column. In each sample, the peroxidasic activity was determined by using hydrogen peroxide, cumene hydroxiperoxide and peroxide dicumene as oxidizing substratum. As shown in Figure 1, in the presence of hydrogen peroxide 7 isoenzymes are evidenced, numbered from I–VII, in the order of elution on the column, When enzymatic reaction is performed in the

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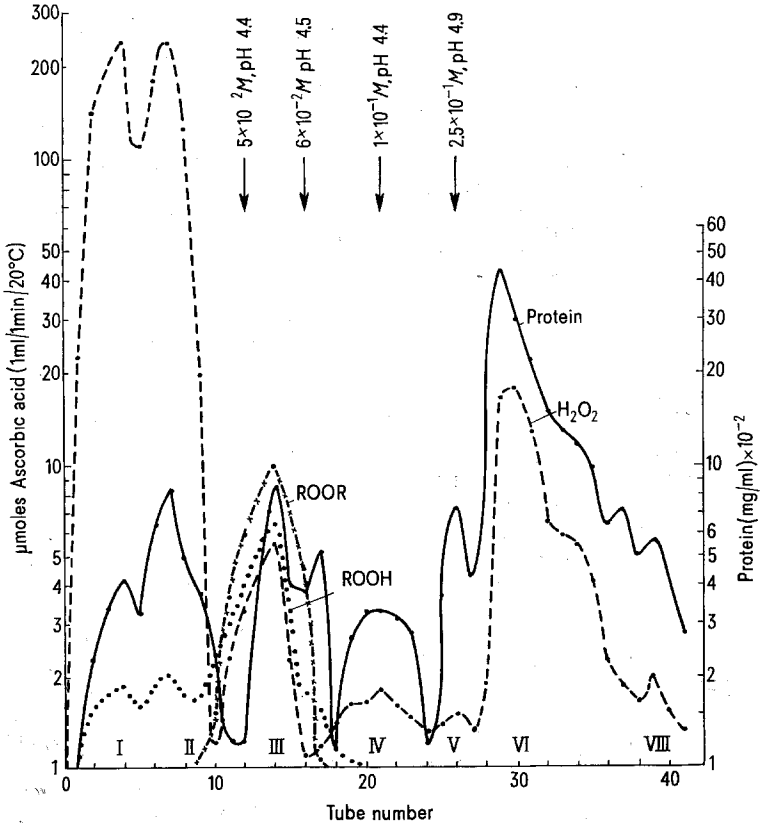

Fig. 1. Separation by chromatography on a CM-cellulose column (1.8 × 19 cm) of a partially purified horse radish peroxidase preparation. Reaction mixture contains: 1 ml 1% peroxidic compound, 0.1 ml 1% ascorbic acid, 2 ml 5×10^{-3} *M* benzidine and 1 ml enzymatic preparation (0.01–0.5 mg/ml)

presence of cumene hydroxiperoxide, only peaks I, II and III are active, while in the case of peroxide dicumene, only peak III is.

The results obtained with chromatographically separated isoenzymes were confirmed with electrophoretic data. As shown in Figure 2, the development of isoenzymes with benzidine and hydrogene peroxide leads to the appearance of 7 electrophoretic bands: 5 cathodic and 2 anodic ones, while in the presence of benzidine, as donor of hydrogen, and of cumene hydroxiperoxide or of peroxide dicumene, 3 and respectively 1 band are evidenced.

From these experimental results it ensues that the real peroxidase oxidating substratum is generally the peroxidic grouping and not only H_2O_2. The 7 chromatographically and electrophoretically separated isoenzymes have different specificities for the 3 oxidating substrata tested. Isoenzyme III decomposes all used peroxidic compounds, evincing maximum activity in the presence of peroxide dicumene (ROOR).

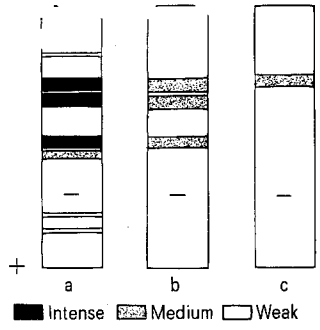

Fig. 2. Variation of the spectrum of horse radish isoperoxidases depending on the oxidating substratum used: a) H_2O_2; b) cumene hydroxiperoxide (ROOH); c) dicumene peroxide (ROOR).

Zusammenfassung. An sieben aus Meerrettichwurzeln isolierten Isoenzymen der Peroxydase konnte substratspezifisches Verhalten nachgewiesen werden.

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Inhibition of Cyclic 3′, 5′-Nucleotide Phosphodiesterase Activity by Diuretics and Other Agents

Adequate intracellular levels of adenosine 3′,5′-monophosphate (c-AMP) are essential for the maintenance of cardiac function[1] but drugs which cause inordinately elevated cardiac c-AMP levels[2] produce necrotic lesions in cardiac tissue[3] and can cause tachyarrhythmias or fibrillation[4,5]. Because cardiac arrhythmias have been reported to result from diuretic therapy and the i.v. administration of mercurial diuretics have been shown to cause ventricular fibrillation[6] the effect of various diuretic agents on cardiac c-AMP phosphodiesterase was investigated.

Methods. Ventricles of female Syrian guinea-pigs were homogenized at 0–4° in 0.01 *M Tris*-HCl buffer pH 7.4 containing 0.25 *M* sucrose. The homogenate was centrifuged at 10,000 × *g* for 10 min at 4 °C, the supernatant decanted, lyophilized and used as the source of c-AMP phosphodiesterase. The preparation was stored at −10 °C and did not lose activity over a period of 6 months. c-AMP phosphodiesterase of rat heart was similarly prepared but not lyophylized. Purified beef heart c-AMP phosphodiesterase was obtained from Sigma Chemical Co.

Platelet phosphodiesterase was prepared according to WOLFE and SHULMAN[7] and the rat brain enzyme according to BROOKER et al.[8].

Enzyme activity was assayed as described in Table I legend and contained sufficient enzyme to degrade 25 to 30% of the substrate and a [H³]c-AMP concentration equal to the Michaelis constant of the enzyme being tested.

Following incubation at 37 °C for 13 min the enzyme was inactivated by heat treatment and the denatured protein was separated by centrifugation. A 10 µl aliquot from each incubation mixture was then applied to cellulose thin layer chromatography sheets and developed in ethanol (95%)-ammonium acetate (1 *M* pH 5.0 containing 0.01 *M* Na_2 EDTA) (70/30). This solvent system separated c-AMP from AMP, adenosine, inosine and hypoxanthine. These purines were produced from AMP due to contaminating enzymes present in some of the enzyme preparations. The c-AMP, AMP and nucleoside areas were cut into small pieces and placed into counting vials containing 15 ml of toluene phosphor solution[9].

Results. The effects of several diuretics, organomercurial compounds and sulfhydryl reagents on guinea pig phosphodiesterase activity were investigated and the results are shown in Table I. Of the compounds tested, mersalyl, meralluride, phenylmercuric acetate, methyl mercuric chloride and PCMB were the most potent inhibitors of the enzyme. Ethacrynic acid, a non-mercurial diuretic was found to be the least effective inhibitor.

Preincubation of the enzyme preparation in the presence of some of the test drugs resulted in greater enzyme inhibition than with no preincubation. Combinations of several concentrations of the 2 mercurial diuretic drugs with theophyl-line resulted in greater inhibition of the enzyme activity than with the diuretics alone. This observation may be of clinical importance because commercial preparations of both mersalyl and meralluride are formulated with large amounts of theophylline to prevent breakdown of the organo-mercurial complex.

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