Another difficulty is that the aflatoxin biosynthesis observed is the result of a number of enzyme activities. These activities may be divided into 2 main phases, a) presumably the formation of an anthraquinone precursor via a polyketide synthetase and b) a cleavage phase involving at least 4 ring-cleaving steps involving enzymes having different substrate specificities[15]. It seems likely that the latter enzymes are induced in response to the appearance of an anthraquinone precursor which in the light of current evidence is probably averufin[16], or a closely related compound[17].

It is, however, clear that protoplasts derived from mycelium of different ages are capable of de novo aflatoxin biosynthesis, this being confirmed by the conversion of labelled ^{14}C acetate to aflatoxin (table 2) and it therefore follows that they must contain the total complement of enzymes required for aflatoxin biosynthesis.

In order to investigate some of these enzyme activities, a number of proven and possible intermediates in aflatoxin biosynthsis were added to the protoplast preparations. Several of the intermediates were converted to aflatoxin while controls containing no added compound did not produce aflatoxin during the same period (table 3). It was observed that protoplasts rapidly take up anthraquinone from the stabilizer-buffer solution as they became stained a yellow-orange colour in the presence of anthraquinone precursors with a corresponding loss of colour from the solution. It was shown that penetration of the membrane had occurred by adding versicolorin A to a suspension of protoplasts which were then centrifuged. The resultant protoplast pellet was washed with buffer-stabilizer, lysed by freezing and thawing in buffer (pH 5.8), and the membrane fraction was centrifuged down; 35% of the versicolorin A that had been added was present in the supernatant fraction indicating that it may pass into the protoplasm.

Work in this laboratory is currently being carried out with lysed protoplasts and preliminary experiments show that they are suitable for preparing cell-free extracts of *A. flavus* capable of carrying out several of the steps involved in aflatoxin biosynthesis.

15 R. Thomas, in: Biogenesis of Antibiotics Substances, p. 160. Academic Press, New York 1965.
16 M. Lin and D. P. H. Hsieh, J. Am. chem. Soc. *95*, 1668 (1973).
17 Y. Berger and J. Jadot, Bull. Soc. chim. Belg. *85*, 271 (1976).

A study of the cytoplasmic factors controlling the rate of catalase synthesis in rat liver

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Summary. A new quantitative method for assessment of the biological activity of the cytoplasmic factors-regulators, controlling the rate of catalase synthesis in rat liver on post-transcriptional stage, has been worked out. The nature of these factors has been established.

There are data in the literature concerning the mechanisms of regulation of protein synthesis rate at the post-transcriptional levels in various eukaryotic tissues[1–4]. Studying the mechanism of synthesis of catalase (KF 1.11.16), Uenoyama and Ono[3,4] have shown that in rat liver cells the synthesis of this protein is controlled at the translational level by 2 factors found in the cytoplasm in a soluble state. These authors have found that in a cell-free system one of the factors (F_{inh}) may bind to the catalase-synthesizing ribosomes, thus suppressing the synthesis of this enzyme, whereas the other factor (F_{act}) prevent association of the F_{inh} with polysomes[3,4]. The presents investigation has been aimed at the development of a quantitative method for assessment of the activity and elucidation of the nature of the factors controlling the synthesis of catalase in rat liver.

Materials and methods. Investigations have been carried out in male rats weighing 160–180 g. The factors were isolated according to Uenoyama and Ono[3,4] using ion-exchange chromatography and re-chromatography on DEAE cellulose. The F_{act} and F_{inh} were isolated from the supernatant and pH 5 fraction of rat liver respectively, so that chromatography of the F_{act} was performed on the post-mitochondrial supernatant, whereas prior to isolation of the F_{inh} the pH 5 fraction had been dialysed. Since the factors are extremely labile, all of the procedures on isolation and purification have been performed at 4 °C. Elution of the factors from DEAE-cellulose columns was achieved with buffers containing increasing concentrations of KCl at a flow rate of 10 ml/h. In the preparations of the factors, the concentration of the protein was estimated according to Lowry et al.[5], the SH-group content by the method of amperometric titration[6], the catalase activity by the manganometric method[7] and electrophoretic mobility was assessed in polyacrylamide gels (PAAG). Depending on condition of the experiment, disk-electrophoresis was performed in a 7% PAAG or in linear gradient of PAAG (from 2.5 to 8% acrylamide). The amino acid composition was evaluated by descending paper chromatography.

Certain parameters of the factors controlling the rate of synthesis of catalase in rat liver

Parameters	F_{act}	F_{inh}
SH-groups (μM/mg)	0.174 ± 0.0095	0.106 ± 0.0106
Protein concentration (mg/ml)	0.123 ± 0.015	0.073 ± 0.007
Activity (μM/min/g of liver)	625	200

1 Levy Fleurette, Doctoral thesis, Univ. Paris.
2 K. Uenoyama and T. Ono, Int. J. cancer *9*, 608 (1972).
3 K. Uenoyama and T. Ono, J. molec. Biol. *74*, 439 (1973).
4 K. Uenoyama and T. Ono, J. molec. Biol. *74*, 453 (1973).
5 O. H. Lowry, A. Rosenberg, A. Farr and R. Randall, J. biol. Chem. *193*, 265 (1951).
6 V. V. Sokolovsky, Lab. Delo *8*, 3 (1962).
7 A. N. Bakh, in: Selected works of academician A. N. Bakh, p. 12. Moscow 1937.

Results and discussion. Employing ion-exchange chromatography and rechromatography on DEAE-cellulose, we have isolated 2 fractions from rat liver cytoplasm, which, according to Uenoyama and Ono[3,4], contain the factors F_{act} and F_{inh}. In the first series of experiments, the biological activity of the preparations was estimated on the basis of their ability to stimulate or suppress the rate of catalase synthesis in rat liver. When measuring the activity of the factors in vitro, as reported by Uenoyama and Ono[3,4], major difficulties are to be overcome, such as isolation of individual mRNA for the catalase and performing synthesis in a cell-free system. We have developed a rather simple and reliable method, which allows for carrying out analyses in vivo in contrast with the model experiments involving cell-free system assays. The principle of the method proposed is in administration of 3 amino-1,2,4 triazole (AT) i.p., this results in a blockage of all tissue catalases, and further increase in the activity of the enzyme with time is attributable only to its synthesis de novo[8–12]. This thesis is based on the results of Price et al.[8] and Margoliash et al.[12].

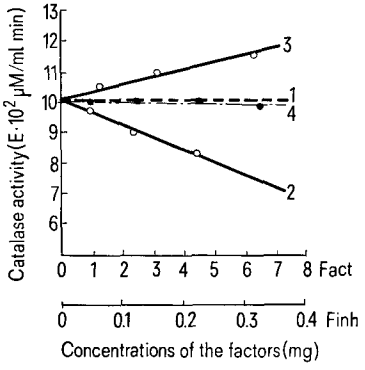

Fig. 1. Assay of the F_{act} and F_{inh} activities. Curve 1: Control (an increase in the catalase level 48 h after administration of AT). Curve 2: An increase in catalase with administration of F_{inh} according the following scheme: 24 h after injection of AT in rat tail vein, a certain amount of the F_{inh} was injected and after another 24 h, the increment of catalase was estimated; each point represents an average of 3 experiments, measuring the level of catalase after administration of the same amounts of the F_{inh}. $tg\alpha_1$: The measure of the activity of the F_{inh}. $tg\alpha_1$ represents the formation of a certain number of catalase activity units per unit time under the action of 1 mg of the F_{inh}. Curve 3: An increase in catalase upon administration of the F_{act}. The factor was given according the scheme depicted above. $tg\alpha_2$ is the measure of the activity of the F_{act}. Curve 4: Catalase activity upon administration of F_{inh} after incubation at 10 °C for 1 h with crystalline trypsin (Ferak).

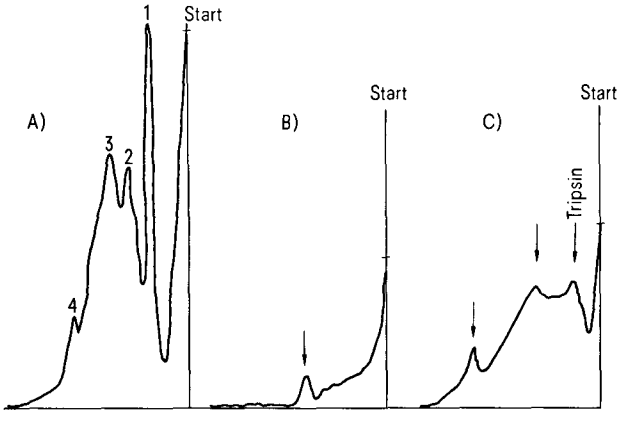

Fig. 2. Densitographs of the factors. *A* The densitograph of the activating factor (after rechromatography on DEAE-cellulose). Electrophoresis (7% polyacrylamide gel) was performed at the current intensity of 2.5 mA–5 mA/gel at 140 V for 2.5 h, using trisglycine electrode buffer, pH 8.3. *B* Densitograph of the inhibiting factor (after rechromatography on DEAE-cellulose). Electrophoresis in linear gradient PAAG (from 2.5 to 8% acrylamide) was carried out at the current intensity of 2.5 mA–5 mA/gel at 300 V for 4 h, using tris-glycine electrode buffer, pH 8.3. *C* Densitograph of the inhibiting factor after treatment with trypsin (Ferak) at 10 °C for 1 h. Electrophoresis in lineat gradient PAAG (from 2.5 to 8% acrylamide) was performed at 2.5 mA–5 mA/gel at 300 V for 4 h, using tris-glycine electrode buffer, pH 8.3.

Price et al.[8] by using separately and in common of 3-amino1,2,4-triazole (AT) and allylisopropylacetamide (AIA), showed that AT inactivates catalase irreversibly, without interfering with its resynthesis, while AIA blocks synthesis of a new catalse without influencing the activity of the catalase already formed[8,9]. It was found too that after the administration of AT, the return of catalase activity is paralleled by a corresponding uptake of Fe^{59} into the catalase[10–12]. Margoliash et al.[12] showed that aminotriazole binds to the protein moiety of catalase to form an irreversible complex.

The present data indicate that the return of catalase results from the formation of new enzyme, but not in consequence of a simple progressive reversal of the inhibitory process that caused the initial disappearance of catalase activity. By estimating the amount of the appearing units of the catalase activity with time, one can assess the effect of the factors upon catalase synthesis when these are administered i.v. after AT. In preliminary experiments, we have calculated the quantity of the enzyme synthesized over a specified time interval. In the present investigation, a control value was considered to be the number of catalase units synthesized during 48 h since administration of AT. This value was 1020 units. In 24 h following administration of AT through tail veins, the rats received 0.2, 0.6 and 1.2 ml of the F_{act} and F_{inh} prechromatographed on DEAE-cellulose. 24 h following administration of the factors, the activity of catalase was estimated in liver cytosol fraction. The activity of the factors was evaluated by graphical methods based on the concentrations of the preparations given to the animals and respective catalase activities assayed. A tangent of the slope of the curves obtained may serve as a measure of the activities of the factors concerned (figure 1). It follows from the data obtained that administration of F_{act} stimulated the rate of catalase synthesis with time, whereas F_{inh}-suppresses it. A ratio of F_{act}-F_{inh} was 3.14 since the activity of F_{act} per g of liver is considerably higher that of the F_{inh}. Homogeneity of the F_{inh} fraction has been demonstrated by disc electrophoresis in polyacrylamide gels (figure 2B). As for the F_{act}, this proved to be a heterogeneous fraction and was separated into 4 fractions (figure 2A). We failed to elucidate whether the heterogeneity of the preparation is the result of nonexhaustive purification of the preparation, or whether there is a spectrum of isoproteins present comprising the biologically active substance. Active preparations, however, have been conclusively shown to be proteins. Thus, in the F_{inh} hydrolysate we could find serine, asparaginic acid

8 V. Price and M. Rechceigl, Fedn Proc. *19*, 49 (1960).
9 V. Price and M. Rechceigl, Nature *189*, 62 (1961).
10 V. Price, W. R. Sterling, V. A. Tarantola, R. H. Hartly and M. Rechceigl, J. biol. Chem. *237*, 3468 (1962).
11 M. Rechceigl and V. Price, Prog. exp. Tumor Res. *10*, 112 (1968).
12 E. Margoliash, A. Novogrodsky and A. Schejter, Biochem. J. *74*, 339 (1960).

and lysine, whereas in the F_{act} hydrolysate we found lysine, asparaginic acid, glycine, serine, glutamic acid, threonine, alanine, phenylalamine, leucine and isoleucine, tryptophan, methionine and valine. The protein nature of the factors is also confirmed by their extremely high lability: even at 4° they lose most of their activity in several h; these factors were then incubated at 60 °C for 1 h, consequently their biological activity disappeared completely in each case (figure 1 shows that catalase activity after administration of F_{inh} subjected to heat inactivation practically coincides with control curve 1). It can been seen that the catalase activity was falling after treatment of F_{inh} with trypsin, too (figure 1, curve 4). Moreover, the densitograph of the inhibiting factor changed, too (figure 2C). In all probability, it is caused by the destruction of the factor by trypsin. These data seem to establish the destructive effect of trypsin on F_{inh}, which shows, in turn, the protein nature of the latter. The high concentration of the SH-groups per g protein is also worthy of note (table).

Thus, the substances isolated from rat liver cell cytoplasm are proteins and are biologically active preparations, which either stimulate or suppress the rate of catalase synthesis in liver.

The pH-dependence of glucose transport inhibition by local anesthetics in human erythrocytes[1]

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Summary. Local anesthetics vary in inhibition of glucose transport in human erythrocytes at different pH-values in the incubation media.

Diverse biological reactions are influenced by local anesthetics[2–4]; among such processes, the glucose transport in human erythrocytes is inhibited by different kinetics, depending on the local anesthetics applied[5]. The molecular mechanism of their action is not yet clarified; neither it is known whether local anesthetics are effective in the charged or uncharged form[3,6]. In this study, the influence of pH (i.e. of the relation between the charged and the uncharged form) on the inhibition of the glucose transport by local anesthetics is investigated.

Material and methods. Human blood was collected in ACD solution (11 g sodium-citrate, 35 g glucose, 4 g citric acid with aqua bidest. ad 1000 ml). The erythrocytes were preloaded by 4 washings with isotonic NaCl solution, containing 200 mM glucose. The further preincubation of the cells is described in the various experiments: 150 µl of these preloaded cells were incubated for 5 sec at 20 °C in 10 ml phosphat-buffer of the desired pH with 0.038 mM C^{14}-glucose and local anesthetics of the concentrations as indicated in figure 1. The further procedure used for the incubation and the analytical methods were described in previous papers[7,8]. The inhibition constants were calculated from the equation:

$$K_i = \frac{[I]}{\frac{v_0}{v_i} - 1} \quad \text{(under the condition } [S] \ll K_m)^9,$$

where v_0 = velocity of the non-inhibited glucose uptake
v_i = velocity of the inhibited glucose uptake
[S] = glucose concentration in the medium
[I] = concentration of the localanesthetic
K_m = Michaelis constant of the carrier-glucose complex

The relative inhibition 'i' in percent was calculated from

$$i = \left(1 - \frac{v_i}{v_0}\right) 100.$$

The derivation of the parables was carried out by a graphic method using a mirror ruler[10].

Results and discussion. As described previously, relative inhibition of glucose transport by local anesthetics at pH 4 was smaller compared with that found at higher pH-values[5]. Inefficiency of the charged forms, increasing with the decrease of pH-value, might be due to their

1 The authors thank Dr G. Zimmer for valuable discussions. The work was supported by the Deutsche Forschungsgemeinschaft.

2 J. M. Ritchie and P. Greengard, A. Rev. Pharmac. *6*, 405 (1966).
3 I. Ueda, H. Kamaya and H. Eyring, Proc. Nat. Sci. USA *73*, 481 (1976).
4 H. Kunze, N. Nahas, J. R. Traynor and M. Wurl, Biochim. biophys. Acta *441*, 93 (1976).
5 L. Lacko, B. Wittke and I. Lacko, J. cell. Physiol. *92*, 257 (1977).
6 A. G. Lee, Nature *262*, 545 (1976).
7 L. Lacko, B. Wittke and P. Geck, J. cell. Physiol. *80*, 73 (1972).
8 L. Lacko, B. Wittke and P. Geck, J. cell. Physiol. *86*, 673 (1975).
9 L. Lacko, B. Wittke and I. Lacko, J. cell. Physiol. *90*, 161 (1977).
10 W. Gellert, H. Küstner, M. Hellwich, H. Kastner, Grosses Handbuch der Mathematik, p. 419. Buch und Zeit Verlagsgesellschaft mbH, Köln 1969.

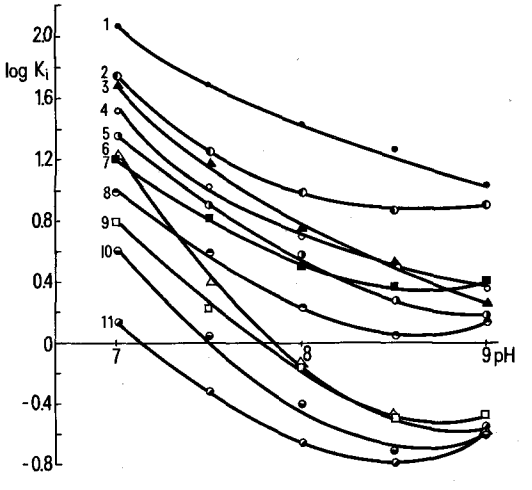

Fig. 1. The logarithmic dependence of the dissociation constants(K_i) of the local anesthetics on the pH. Erythrocytes were preloaded with 200 mM glucose and incubated for 5 sec at 20 °C in phosphat-buffer at pH 7.9, containing C^{14}-glucose and the local anesthetics in the indicated concentrations. *1* 0.5 mM brufacaine, *2* 15 mM mepivacaine, *3* 12 mM procaine, *4* 8 mM lidocaine, *5* 6 mM hostacaine, *6* 4.5 mM ultracaine, *7* 1.5 mM tetracaine, *8* 3 mM baycaine, *9* 1.6 mM butacaine, *10* 1.2 mM oxybuprocaine, *11* 0.35 mM dibucaine. (The concentrations used are the same as in our previous paper[5].)