of the agonist-receptor complex. A plot of 1/[A] against 1/[A'] gives a straight line with a slope of 1/q, and the K_A-values are equal to (slope-1) divided by the intercept of the line on the 1/[A]axis. Experimental values for 1/[A] and 1/[A'] were fitted by a least-squares technique to the equation ($r = 0.974 - 0.998$). The relative intrinsic efficacies of compounds **I–IV** in relation to that of acetylcholine were determined according to the method of Furchgott and Bursztyn[8]: relative intrinsic efficacy $= e_x/e_{Ach}$.

Results and discussion. The following immediate observations can be made upon examination of the data in the table. The differences in muscarinic activity between acetylcholine and compounds **I–IV** are due to differences in the affinities to the receptor as well as differences in the efficacies. Both the sulfonium analogues **III** and **IV** possess a higher intrinsic efficacy than acetylcholine. This is the reason why their relative affinities are smaller than the corresponding relative activity values. In the case of the 2 ammonium anlogues **I** and **II**, values for relative affinities are higher than the corresponding relative activities, since these 2 drugs show lower values for their relative intrinsic efficacies than acetylcholine. However, the data in the table show some parallelism between relative activities and relative affinities, since the differences in relative intrinsic efficacies of acetylcholine and **I–IV** are not too large.

The ratio K_A/ED_{50} varies for acetylcholine and **I–IV** from 232 to 37. Data obtained by various workers[13] have shown that, with increasing potency of muscarinic agonists, the ED_{50} becomes progressively smaller than the calculated K_A-value. With the exception of the sulfonium compound **IV**, this is true for the other derivatives under investigation. Compound **III** with the highest potency possesses also the highest K_A/ED_{50} ratio. The less active ammonium derivatives show lower values for K_A/ED_{50}.

Until recently the knowledge of the mechanism of how muscarinic agents act on their receptors has been derived from an analysis of the actions of agonists on certain pharmacological responses, such as the contraction of smooth muscle and the decrease in contractile amplitude of heart muscle, respectively[11]. Quantitative parameters describing the results of such experiments are the ED_{50}, K_A-values and the efficacies, shown in the table. With the development of tritium-labelled muscarinic ligands of high specificity and high specific activity, one can now measure directly the binding of muscarinic agents to their receptors. The point of interest to emerge from these binding studies is that the binding curves respresent binding to 2 independent sites for which agonists have different affinities[11,14]. There exist 2 classes of agonist binding sites, one having a high affinity constant (K_H) and the other a low affinity constant (K_L) for agonist binding. Comparing the parameters derived from binding studies with those derived from analysis of pharmacological experiments, certain relationships emerge. There is a positive correlation between K_H and ED_{50}[14]; moreover, the absolute values tend to be similar. Values of K_L are close to the dissociation constants K_A. The question arising is, which binding site is coupled to the pharmacological response? At present, it seems to be the low affinity site K_L[11]. If this is true, the action of muscarinic agonists can be described with 2 parameters obtained in pharmacological experiments: intrinsic efficacy e – there is no capability for determining e in a radioligand procedure – and the dissociation constant K_A.

1 Structure and conformation activity relationships of heterocyclic acetylcholine analogues, VIII.

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Protein synthesis inhibition induced by dimethylnitrosamine and diethylnitrosamine on isolated rat hepatocytes

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Summary. Time- and dose-dependent protein synthesis inhibition takes place following exposure to high doses of dimethylnitrosamine (DMN) or diethylnitrosamine (DENA) in isolated rat hepatocytes. The ability of DENA to depress protein synthesis is 5-fold higher than that of DMN. Cells inhibited by 60 min exposure to DMN or DENA, and then incubated in a nitrosamine-free medium, regain their initial rate of protein synthesis. This recovery is faster and more complete for DENA-treated cells.

The i.p. injection of a single high dose of DMN or DENA in rodents produces an early and severe depression of protein biosynthesis in the liver[1,2]. Knowledge of the mechanism underlying this effect is inadequate to determine whether the nitrosamines act by inactivating some essential component(s) of the protein synthesis machinery or by interference with regulatory functions[3,4].

As a tool for studying the forward protein synthesis inhibition produced by DMN and DENA, we have used isolated rat hepatocytes prepared by an enzymatic perfusion technique[5,6], overcoming in this way many of the difficulties arising in the in vivo experiments. The viability of isolated rat liver cells is not impaired within 2 h of incubation by high doses of DMN or DENA. A time- and dose-dependent protein synthesis inhibition takes place in isolated hepatocytes treated with DMN or DENA: this effect is

much more relevant for DENA. By contrast, the protein synthesis resumption is faster and larger in cells previously inhibited by exposure to DENA than in cells treated with equally toxic doses of DMN.

Materials and methods. DMN was purchased from Eastman Kodak Co., USA, and DENA from Merck-Schuchardt Co., Federal Republic of Germany. Both drugs were redistilled before use. Male Sprague-Dawley rats weighing 150–200 g each were used. Under ether anesthesia, the liver was excised and then perfused, via portal cannulation, with collagenase and hyaluronidase. Isolated cells were filtered through a nylon mesh and further purified by 3 steps of low speed centrifugation at 4 °C[5,6]. By this procedure about $1.5 \cdot 10^8$ purified hepatocytes were obtained from a single animal. A cell viability of about 90% was found by trypan blue exclusion test. Incorporation experiments were carried out in samples of $2 \cdot 10^6$ cells/ml in suspension buffer (containing 100 units/ml of ampicilline) supplemented with a high concentration (4× normal) of 'cold' amino acids and with 2.5 µCi/ml of ^{14}C-protein hydrolysate (Amersham, England, sp. act. – 50 mCi/milliatom of carbon). This high concentration of 'cold' amino acids was used to avoid uncontrolled fluctuations in the specific activity of radioactive precursors[7]. In fact, the high rate of protein degradation occurring in isolated hepatocytes[8] may cause a not negligible isotope dilution. In order to attain equilibrium between the intracellular amino acid pool and this high concentration of amino acids in external medium, cells were pre-incubated for 20 min at 37 °C. DMN or DENA was then added at appropriate concentrations; and at progressive time-intervals 1 ml aliquots were withdrawn, precipitated with cold 10% trichloroacetic acid and processed for acid-insoluble radioactivity. Results were expressed as cpm/mg of protein.

The intracellular ATP content was determined, after perchloric acid extraction, by the Boehringer ATP-UV method. Lactic dehydrogenase (LDH) released in incubation medium was measured by the spectrophotometric procedure[9].

Results. Preliminary controls by the dye exclusion test showed that the percentage of viable hepatocytes does not appreciably decrease during exposure to high doses of DMN or DENA.

In order to attain a better characterization of the functional conditions of isolated hepatocytes during prolonged incubation in the presence of nitrosamines, we have determined at different time-intervals the level of intracellular ATP and the amount of LDH released into the external medium. As shown in figure 1, A, the ATP concentration sharply decreases when cells from ice-cold medium are transferred into a 37 °C bath and incubated for 20 min. At this time, nitrosamines were added, and the incubation of treated and control cells was continued in parallel up to 3 h. During this time, no further decrease was observed in intracellular ATP concentration either in control or in nitrosamines-treated samples: therefore, the exposure to high doses of DMN or DENA do not appear to modify the energetic state of isolated hepatocytes. It has been reported that LDH leakage is indicative of a loss of functional integrity of cell membrane[10]. In figure 1, B it is evident that the amount of LDH in the external medium slowly increased with time, showing that cellular membrane were progressively im-

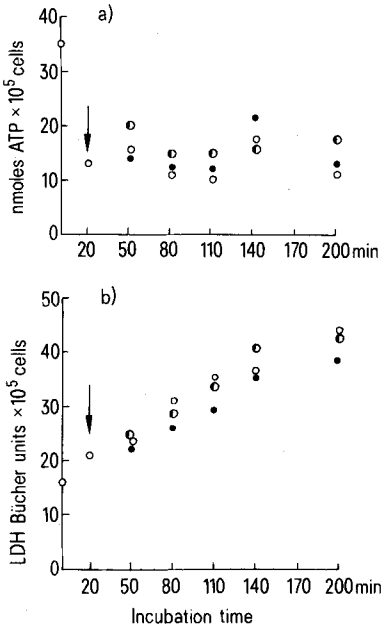

Fig. 1. *A* Intracellular ATP content in untreated control hepatocytes (○—○) and in hepatocytes incubated in presence of 50 mM DMN (●—●) or in 10 mM DENA (◐——◐). *B* Time-course of LDH releasing in the incubation medium by untreated control hepatocytes (○—○) and by hepatocytes incubated in presence of 50 mM DMN (●—●) or 10 mM DENA (◐——◐). Isolated hepatocytes ($2 \cdot 10^6$ cells/ml) were incubated at 37 °C in a complete medium, lacking only radioactive amino acids. After 20 min (arrows) nitrosamines were added and incubation continued up to 200 min. At increasing time-intervals, 5 ml aliquots were withdrawn and cells pelletted and extracted with cold 0.5 N perchloric acid. The amount of ATP in the extract was determined by a spectrophotometric method (Boehringer ATP-UV-test). LDH concentration in the supernatant was determined according to Bergmeyer[5].

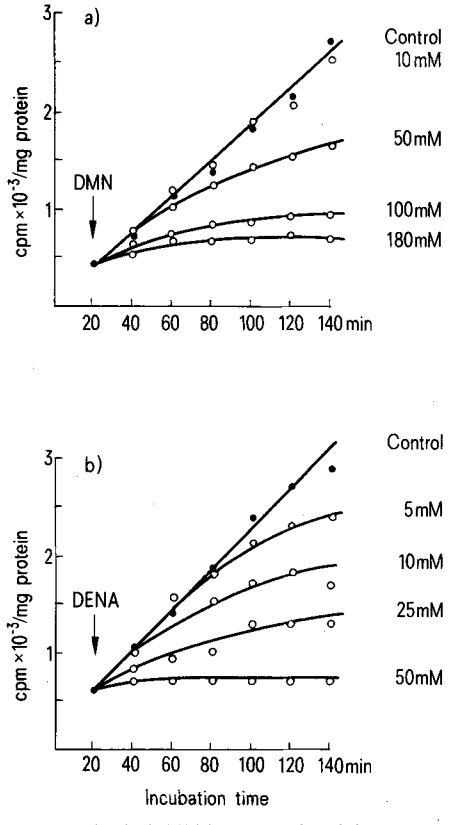

Fig. 2. Protein synthesis inhibition in isolated hepatocytes treated with different doses of DMN (A) or DENA (B).
Arrows indicate when drugs were added (after 20 min of preincubation, needed for attaining equilibrium between intracellular amino acid pool and the external medium). Experimental details are described in methods.

Table 1. Comparative inhibition of protein synthesis following 60 min treatment with increasing concentration of DMN and DENA

Treatment	Concentrations				
	10 mM	25 mM	50 mM	100 mM	180 mM
Dimethyl-nitrosamine	0%	12%	24%	58%	75%
Diethylnitrosamine	27%	69%	91%	-	-

Table 2. Synthetic activity (percent of controls) of hepatocytes incubated for 60 min at 37 °C in presence of 100 mM DMN or 25 mM DENA and then reincubated in a drug-free medium

Treatment	Reincubation time (min)			
	0	30	60	120
Dimethylnitrosamine	65	79	78	78
Diethylnitrosamine	67	84	90	96

paired during incubation. No additive damages due to nitrosamines are apparent; LDH levels in control and in treated samples correspond closely.

The time-course of protein synthesis in isolated hepatocytes exposed to increasing doses of DMN (figure 2, A) or DENA (figure 2, B) was then determined. As shown in the figures, treatment with both nitrosamines results in a definite dose-dependent inhibition in the incorporation of labelled amino acids into acid-insoluble material. The effectiveness of the 2 drugs is not the same: at 10 mM concentration DMN fails to depress isotope incorporation, while a- marked reduction is already detectable at 5 mM concentration of DENA.

The difference in the effectiveness of the two nitrosamines is pointed out in table 1, which reportes the percentage of protein synthesis inhibition attained after 60 min exposure to equal doses of DMN or DENA. At all concentrations used, the inhibition caused by DENA is about 5 times higher than that of DMN.

Details of an experiment to test whether hepatocytes inhibited by treatment with nitrosamines are able to restore their initial rate of protein synthesis when reincubated in a fresh nontoxic medium, are reported in figure 3. Results (summarized in table 2) show that after exposure to equally toxic doses of the 2 nitrosamines, the inhibited cells progressively recover their ability to synthetize proteins. Immediately after 60 min of treatment with 100 mM DMN, the rate of protein synthesis is reduced to 65% of control cells; this value increases to about 80% after 30 min of incubation in the drug-free medium, but not further increase occurs for longer incubation time. Cells exposed to 25 mM DENA, however, show a greater ability to restore their protein synthesis. In fact, from an initial 67% residual activity, an increase to 84% was reached after 30 min, and the recovery progressively increased up to 96% after 2 h of reincubation.

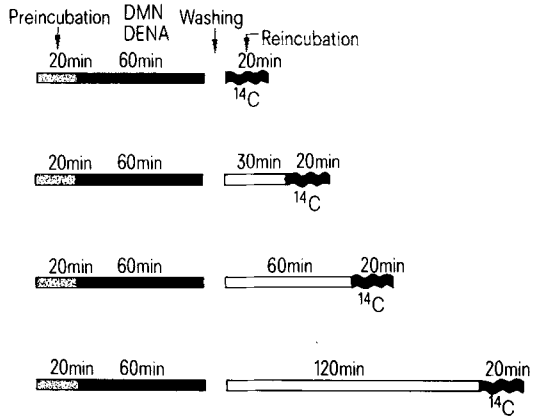

Fig. 3. Rate of protein synthesis in isolated rat liver cells incubated for 60 min at 37 °C in presence of DMN (100 mM) or DENA (25 mM) and then reincubated for increasing times in a fresh, drug-free medium.

After different times of recovery, radioactive amino acids were added and cells further incubated for 20 min. Incorporation values, expressed as residual activity (percent of controls) are reported in table 2.

Discussion. Nitrosamines such as DMN and DENA, are known to display their toxicity in target organs only after metabolic activation[2]. Their inhibiting effect on protein synthesis indicate that the isolated hepatocytes retain the ability to metabolize nitrosamines into active intermediates. In addition to the effects induced by toxic metabolites, the effects of their powerful protein-denaturing properties must be considered[11]. These effects may be negligible in the liver of intact animals because of the rapid dilution of the drugs, but their action cannot be excluded in isolated liver cells suspension, because hepatocytes are exposed for relatively long times to high concentrations of drugs. Since DENA is a more powerful denaturating agent than DMN, the higher inhibiting effect of this drug might be due, at least in part, to this denaturating action[12].

However, both the absence of damages on cellular membrane, as revealed by the LDH-leakage test, and the constance in the intracellular ATP content, argue against the occurrence of relevant damages due to protein denaturation.

It is clear that in order to understand the differences in the dose-effect relationship between DMN and DENA, comparative data are needed on the rate of their metabolic conversion in isolated hepatocytes. Experiments are in progress to clarify this point.

Isolated hepatocytes surviving in vitro retain their metabolic characteristics and their responsiveness to enzymic induction[13]; they also retain their ability to recover the initial rate of protein synthesis after being inhibited by nitrosamines. For these reasons, the use of these cells may be a very suitable tool, in place of the cell-free systems or of the liver slices, in attaining a definite characterization of the protein synthesis inhibition induced by nitrosamines.

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