any effect on the spontaneous activity of mice. In *in vitro* studies hydrotrichlorothiazide did not exhibit any antihistaminic, anticholinergic, or antispasmodic activity. Hydrotrichlorothiazide had one-half the carbonic anhydrase inhibiting activity of chlorothiazide and five times that of hydrochlorothiazide (SHEPPARD).

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Research Department, CIBA, Summit (New Jersey), February 9, 1960.

Zusammenfassung

In der Reihe der Dihydro-benzothiadiazine wurde mit Hydrotrichlorthiazid (III) ein neues, ausserordentlich stark wirksames Diuretikum gefunden. Herstellung und pharmakologische Evaluation werden kurz beschrieben. Im Vergleich zu Hydrochlorthiazid (I) ist III beim Hund *per os* bis zu 20mal stärker diuretisch wirksam.

Effect of Methanol and Dioxan on the Action of Chymotrypsin on L-Phenylalanine Methyl Ester

Many studies on the kinetics and specificity of chymotrypsin have been carried out in methanol-water mixtures because of the limited solubility in water of the synthetic substrates employed in these studies[1–4]. Thus, SNOKE and NEURATH[4] investigated the action of chymotrypsin on benzoyl-L-phenylalanine methyl ester in a system containing 30 vol.% of methanol. The present paper deals with the effect of methanol and dioxan on the action of chymotrypsin on non-benzoylated L-phenylalanine methyl ester which, like the corresponding ethyl ester[5], is readily hydrolyzed by chymotrypsin in aqueous solution.

Worthington crystalline, salt-free chymotrypsin was used. Its action was followed by measuring the disappearance of the ester, using Hestrin's hydroxamic acid method[6]. Each reaction mixture contained, in 5 ml 0·04 *M* phosphate buffer: chymotrypsin, 0·2 mg; substrate, approximately 50 μmoles (L-phenylalanine methyl ester) or 25 μmoles (benzoyl-L-phenylalanine methyl ester). The temperature was 30°C. One ml samples were tested by Hestrin's method (15 min treatment with hydroxylamine prior to the addition of HCl and $FeCl_3$). The results of the experiments are summarized in the Table. The figures in the Table represent the Klett-Summerson colorimeter readings (Filter 54). Blanks run simultaneously without addition of enzyme gave, at the end of the experiments, values which were equal or very close to the initial values.

As shown in the Table, the action of chymotrypsin on L-phenylalanine methyl ester was completely inhibited in the presence of 30 vol.% of either methanol or dioxan. Under the same conditions, benzoyl-L-phenylalanine methyl ester was readily hydrolyzed. The Table also shows that 15 vol.% of methanol very strongly inhibited the action of chymotrypsin on L-phenylalanine methyl ester, and that even 7·5 vol.% caused a strong inhibition.

Effect of methanol and dioxan on the action of chymotrypsin

Substrate	Medium	pH	Time, min		
			0	10	20
PME*	Water	7·5	370	180	50
PME*	Methanol 7·5%	7·5	370	325	260
PME*	Methanol 15 %	7·5	380	365	340
PME*	Methanol 30 %	7·5	385	385	375
PME*	Dioxan 30 %	7·5	375	375	365
BPME**	Methanol 30 %	7·5	365	20	15
BPME**	Methanol 30 %	7·5	360***	120***	30***
BPME**	Dioxan 30 %	7·5	365	25	15
BPME**	Dioxan 30 %	7·5	375***	120***	30***
PME*	Water	6·5	390	175	50
PME*	Methanol 30 %	6·5	420	415	415
BPME**	Methanol 30 %	6·5	395	160	90

* L-Phenylalanine methyl ester hydrochloride.
** Benzoyl-L-phenylalanine methyl ester.
*** With half the amount of enzyme.

The findings here reported on the complete inhibition of the action of chymotrypsin on phenylalanine methyl ester by methanol or dioxan under conditions where the corresponding benzoyl derivative was readily hydrolyzed, may be of interest and deserve a closer investigation. In a previous communication[7] it was suggested that chymotrypsin does not hydrolyze the phenylalanine ester directly, but first converts it, by a transfer reaction, to a dipeptide ester (or to an ester of a higher peptide), and that this compound, bearing a 'secondary peptide bond' is then rapidly hydrolyzed by the enzyme. Since methanol or dioxan did not, in our experiments, prevent the hydrolysis of the benzoyl derivative of the ester, this assumption, if correct, would mean that both organic solvents inhibit the primary transfer reaction.

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Zusammenfassung

Die Spaltung von L-Phenylalanin-methylester durch Chymotrypsin ist in Gegenwart von 30 Vol.% Methanol oder Dioxan vollständig gehemmt, während unter denselben Bedingungen Benzoyl-L-phenylalanin-methylester intensiv hydrolisiert wird.

[1] S. KAUFMAN, H. NEURATH, and G. W. SCHWERT, J. biol. Chem. *177*, 793 (1949).
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Incorporation of S^{35}-Methionine in the Microsomes and Soluble Proteins During the Early Development of the Sea Urchin Egg

Previous work from this Laboratory has shown that S^{35}-methionine given to unfertilized eggs of *Paracentrotus lividus* is stored entirely in the so-called non-protein fraction (fraction soluble in cold 10% trichloroacetic acid) and largely converted into glutathione[1,2].

[1] E. NAKANO and A. MONROY, Exp. Cell Res. *14*, 236 (1958).
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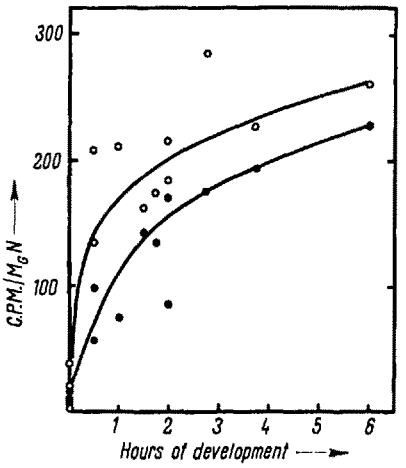
Upon fertilization or parthenogenetic activation the radioactivity of the non-protein fraction rapidly decreases while at the same time a progressive labeling of the mitochondria was found to take place[1,3]. It could be shown, however, that during the first 3–4 h of development the radioactivity in the mitochondria is confined to low molecular material and it is only after this time that a progressive labeling of the mitochondrial proteins occurs[4]. The study of the uptake of the labeled methionine by the microsomes and the soluble proteins has been made possible only recently by the availability of an ultracentrifuge. This investigation was also stimulated by the results being obtained at the same time in our laboratory on the effect of ethionine (an analogue of methionine) on the differentiation of the sea urchin eggs (Bosco and MONROY, in press). These experiments, in fact, suggested that some protein(s) which play an important rôle in the differentiation of the primary mesenchyme are being synthesized very early in the course of development, viz. during the first 3 h after fertilization, although they are called into action only when the differentiation of the primary mesenchyme takes place.

In the present experiments the DL-S^{35}-methionine (Radiochemical Center, Amersham) was incorporated in the unfertilized eggs of *Paracentrotus lividus* using the technique of NAKANO and MONROY[5]. 10 μC of carrier-free DL-S^{35}-methionine were injected into each animal and 2 h later the eggs were collected (by shaking the gonads in sea water) and freed of tissue debris by filtration through cheesecloth. After several washings with sea water, the eggs were fertilized and allowed to develop or collected unfertilized. The jelly-coat was removed by the usual treatment with acidified sea water (at about pH 5). The eggs were homogenized in ice-cold 0·44 *M* sucrose in 0·1 *M* citrate buffer at pH 6·4 using a glass homogenizer with a motor-driven teflon plunger. The homogenate was centrifuged at 23·500 *g* for 15 min in a Serval superspeed refrigerated centrifuge to remove cell debris and mitochondria. The supernatant was then submitted to an ultracentrifugation at 105·000 *g* for 60 min in a model *L* Spinco ultracentrifuge. The sedimented microsomes were resuspended in sucrose (as above) and centrifuged again at 105·000 *g* for 30 min. They were then suspend in distilled water (using a small whole-glass homogenizer) and 0·1 cm^3 samples were plated on aluminium planchets, dried and counted at infinite thiness. 0·1 cm^3 samples ware also taken for N determination (by Nesslerization after combustion). The supernatant of the first ultracentrifugation, i. e. the cytoplasmic fraction after removal of the microsomes, was dialysed against several changes of cold distilled water in order to remove all low molecular material. In the extract of unfertilized eggs a small precipitate forms upon dialysis; its relative amount decreases in the developmental stages and disappears alltogether in the early blastula stage. In the present experiments it was removed by centrifugation and discarded, its analysis being postponed to a later occasion. Some assays showed, however, that its activity is very low. The dialysed extract was then concentrated several times by dialysis against gum arabic and then 0·1 cm^3 samples were used for counting and for N determinations as described above. A thin mica end-window Geiger-Müller counter and an EKCO automatic scaler were used. The specific activities have been expressed as counts *per* min *per* mg total N.

The diagram (Fig.) shows the uptake of the isotope in the microsomes and in the soluble proteins during the first 6 h of development. In the unfertilized egg, as was expected, the activity is megligible in both fractions. An abrupt and considerable uptake is already observed both in the microsomes and in the soluble proteins during the first hour of development, the microsomes being the most rapidly and strongly labeled. It is worth mentioning also that in two experiments in which the eggs cleaved very irregularly and did not develop beyond the morula stage, there was very little, if any, uptake both in the microsomes and in the soluble proteins.



Uptake of S^{35}-methionine in the microsomes (o) and soluble proteins (•) of developing *Paracentrtous lividus*

As has been mentioned already, investigations in this laboratory (Bosco and MONROY, in press) have provided indirect evidence that a methionine requiring protein which plays an important rôle in the differentiation of the primary mesenchyme, is synthesized also during the first few hours after fertilization. Although the final proof of such a synthesis will only be given by the identification of the newly synthesized protein(s) the present results seem to suggest that in the sea urchin egg protein synthesis is started very early in the course of development.

The results of serological investigations [6–9] have suggested that in the sea urchin development the synthesis of new protein species begins rather late. However, it might be that the proteins synthesized earlier have the same antigenic specificity as those of the unfertilized egg and hence cannot be detected as separate antigens.

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A. MONROY

Istituto di Anatomia comparata, Università di Palermo (Italia), October 12, 1959.

Riassunto

Nelle uova di *Paracentrotus lividus* già pochi minuti dopo la fecondazione si inizia una attiva incorporazione di metionina-S35 nei microsomi e nelle proteine solubili. Si avanza l'ipotesi che cio possa indicare l'iniziarsi molto precoce della sintesi di una o più proteine.

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