Amylolytic Enzymes in Dog Serum

Two peaks of amylolytic activity have been obtained from dog serum by fractionating it on Sephadex G-75 column[1]; they had been previously regarded as different molecular forms of amylase, i.e. amylase isoenzymes[1,2].

In the course of our researches on human urinary amylolytic enzymes, we found 2 peaks of amylolytic activity in the effluent fractions from a Sephadex G-100 column[3]. While the second peak showed the typical behaviour of an α-amylase[4], the first one could be further fractionated into 2 enzymes, both differing from α-amylase for some catalytic aspects[5]. In view of these results with human urinary enzymes, we thought it worth while to investigate the dog serum enzymes.

We planned to use dog serum since, as shown by others, dog serum does contain at least 2 amylolytic enzymes which can be separated by means of gel filtration. The reported gel filtration behaviour of dog serum amylolytic enzymes suggested us the possibility that they could bear some relationship with the amylolytic enzymes of human urine. Amylolytic enzymes of human urine are being investigated in our laboratory and, according to our point of view, the enzymic peak which can be separated by means of gel filtration are not to be regarded as amylase isoenzymes, but merely as different kinds of enzymes, on the basis of their catalytic behaviour. The experiments reported in our paper show this to be the case in dog serum too. On the other hand, according to data from others and our unpublished results, only 1 gel filtration peak of amylolytic activity is to be found in human serum. The reasons for the differences between human serum, human urine and dog serum are not, at present, apparent.

Serum obtained from a male dog was dialyzed against 0.14 *M* sodium chloride containing merthiolate (1/10,000) and was applied to a Sephadex G-100 (Pharmacia, Uppsala) column, equilibrated and eluted with the same solution. Enzymic activity was determined in the eluted fractions by means of 3 methods: (1) by incubating with starch at pH 7 and measuring the sugar split off by means of a phenylhydrazine reagent[6]; (2) by incubating with starch at pH 5 in the presence of EDTA (disodium salt) 0.05 *M* and determining the glucose split off by means of a rather specific *Tris*-glucose oxidase-peroxidase reagent (TGO)[7]; (3) by incubating with maltose and determining the glucose, as a product of hydrolysis, with an *o*-toluidine reagent[8].

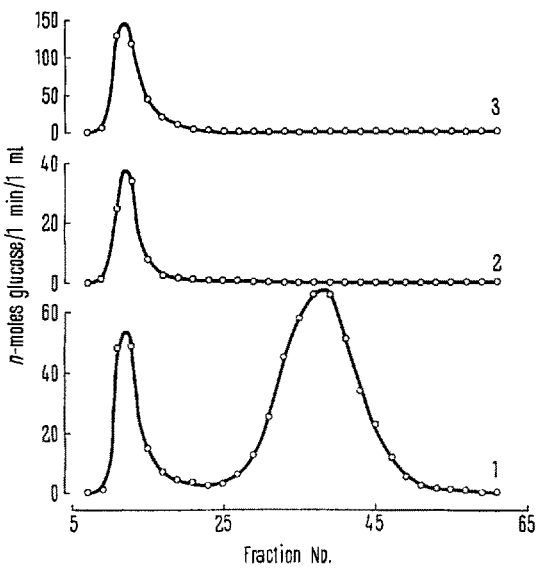

Fig. 1. Distribution of enzymic activity in the fractions from a Sephadex G-100 column. 4 ml of dog serum were fractionated on a 2 · 50 cm column, and the enzymic activity was measured in the eluates with starch as substrate by means of a phenylhydrazine (pattern 1) and a TGO (pattern 2) method, and with maltose as substrate by means of an o-toluidine method (pattern 3).

2 peaks were found with the phenylhydrazine method, while the enzymic activity measured by the 2 other methods was recovered only in the fractions corresponding to the first peak (Figure 1).

The tubes corresponding to the 2 peaks were pooled and concentrated. Starch was incubated with the 2 fractions obtained: ethanol, to a concentration of 50 %, was added and, after centrifugation, the alcohol was evaporated. The resulting solutions were deionized over amberlite MB 3, and the clear solutions obtained were dried, dissolved in the minimum amount of water and examined by means of thin layer chromatography. Silica gel-boric acid plates were prepared according to STAHL[9]: the chromatograms were developed with ethyl acetate-isopropanol-water (3/2/1) and were sprayed with an anisaldehyde reagent[9]. Glucose and maltose were used as

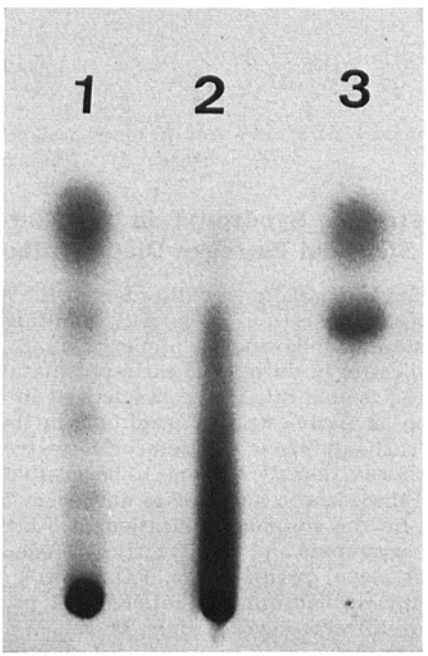

Fig. 2. Thin-layer chromatography of the hydrolysis products from peak I (1) and peak II (2) enzymes; starch was used as a substrate. Glucose and maltose (3) were run in parallel, for comparison.

[1] I. UJIHIRA, R. L. SEARCY and J. E. BERK, Proc. Soc. exp. Biol. Med. *115*, 996 (1964).
[2] J. E. BERK, R. L. SEARCY, S. HAYASHI and I. UJIHIRA, J. Am. med. Ass. *192*, 389 (1965).
[3] C. FRANZINI, Biochim. appl. *12*, 101 (1965).
[4] C. FRANZINI, Boll. Soc. ital. Biol. sper. *52*, 55 (1966).
[5] C. FRANZINI, Quad. Sclavo Diagn. *1*, 430 (1965).
[6] C. FRANZINI, Biochim. appl. *12*, 41 (1965).
[7] A. DAHLQVIST, in *Disorders due to intestinal defective carbohydrate digestion and absorption*. (Ed. P. DURAND; Il Pensiero Scientifico Publisher, Rome, 1964).
[8] C. FRANZINI, Biochim. appl. *12*, 217 (1965).
[9] E. STAHL, *Thin-layer chromatography* (Academic Press, New York and London, 1965).

standards. Maltose, poorly resolved slower-moving saccharides and traces of glucose were found in the incubation mixture of peak II, while almost exclusively glucose and some slower-moving oligosaccharides were detected in peak I (Figure 2). Non-migrating dextrins were present in both cases.

In further experiments the peak I enzyme was incubated with starch, and the glucose split off was measured, at intervals, by means of both the TGO and the phenylhydrazine reagents. When the results were calculated as μmoles of glucose split off, the same amount of sugar was found with the 2 methods, this fact being taken to indicate that the only reducing sugar liberated by enzymic hydrolysis was glucose.

Preincubation with EDTA (0.025 M) caused 95% inhibition of peak II enzyme, but had no appreciable effect on peak I enzyme.

The data reported here show that the 2 enzymes are different for both molecular and catalytic properties. While peak II exhibits some catalytic properties of an α-amylase (α-1,4-Glucan 4-glucanohydrolase, E.C. 3.2.1.1), the peak I enzyme is able to split maltose and hydrolyzes starch by splitting off glucose molecules: therefore it may be regarded as an α-glucosidase (α-D-Glucoside glucohydrolase, E.C. 3.2.1.20) or as a glucoamylase (α-1,4-Glucan glucohydrolase, E.C. 3.2.1.3.). In this respect it bears some relationship with the corresponding enzymes of human urine[10].

Hence, on the basis of the data herein reported, the 2 amylolytic enzymes cannot be regarded as amylase isoenzymes.

Further studies are now in progress in order better to characterize the molecular and catalytic properties of the peak I enzyme.

Riassunto. Mediante gel filtrazione si sono separati due enzimi amilolitici dal siero di cane; mentre il primo mostra alcune proprietà della alfa-amilasi, il secondo attacca l'amido con liberazione di glucosio ed è inoltre capace di idrolizzare il maltosio. I due enzimi pertanto non possono essere considerati come isoenzimi, ma come enzimi differenti.

C. FRANZINI and P. A. BONINI

Laboratory of Clinical Investigations, The Civic Hospital 'S. Antonio Abate', Gallarate (Italy), 25th November 1966.

[10] C. FRANZINI and P. A. BONINI, to be published.

Early-Estrogen Syndrome in the Rat by the Non-Steroidal Estrogen Diethylstilbestrol

A complex of characteristic disorders of the regulatory mechanisms of the estrous cycle, with resulting anovulation and sterility following the application of a sufficient amount of estrogen during the early post-natal development in the female rat, was characterized in this communication as 'early-estrogen' syndrome in the sense of the term 'early-androgen' syndrome of SWANSON and VAN DER WERFF TEN BOSCH[1]. It seems to be possible to induce analogical disorders in addition to androgens and estrogens also by the suitable application of other steroids, such as progesterone and desoxycorticosterone and lastly cholesterol alone (reviewed by TAKEWAKI[2]), although our attempts to confirm this influence of progesterone and cholesterol were unsuccessful[3]. It seemed of interest, therefore, to attempt to induce the early-estrogen syndrome by a non-steroidal estrogen diethylstilbestrol.

19 females and 21 males of our laboratory strain of rats received at the age of 5 days a single s.c. injection of 1.25 mg diethylstilbestrol in 0.1 ml peanut oil. The vaginae opened at the age of 29.3 $\pm$ 0.8 days (S.D. 2.9; 95% confidence interval 27.5–31.1), i.e. significantly ($P < 0.01$) earlier than in the controls (47.8 $\pm$ 1.3 – S.D. 4.3; 44.7–50.9). At 100 days of age, treated females were placed in a cage with normal fertile males and treated males with normal fertile females, in the proportion of 2 males to 3 females, for 3 weeks. All treated animals were entirely sterile. At the age of 150 days the animals were killed and their gonads examined histologically.

Immediately after death the gonads were fixed in Bouin solution and paraffin embedding was employed. Sections were stained by hematoxylin-eosin. In the ovaries numerous primordial, growing, and Graafian follicles were present. Corpora lutea were absent. In some Graafian follicles the oocytes with corona radiata were free in antrum. A considerable finding were papillae of connective tissue penetrating into the granulosa cell layer (Figure 1). The interstitial cells among the follicles were hyperplastic and hypertrophied. In the testicles

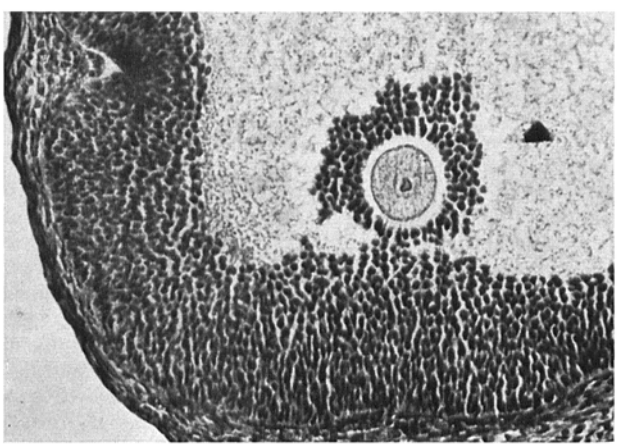

Fig. 1. Ovary of a rat with early-estrogen syndrome by diethylstilbestrol. Detail of a large Graafian follicle. On the left a characteristic fold in the granulosa layer due to a papilla of the theca interna. × 200.

[1] H. E. SWANSON and J. J. VAN DER WERFF TEN BOSCH, Acta endocr., Copenh. *45*, 1 (1964).
[2] K. TAKEWAKI, Experientia *18*, 1 (1962).
[3] Unpublished results (1964).