INACTIVATION OF SECRETIN AND CHOLECYSTOKININ CONCENTRATES BY BODY FLUIDS AND ENZYMES

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ABSTRACT.—Secretin concentrates underwent inactivation on incubation at 37°C. with dog, rat and horse sera, canine plasma protein fractions, splenic and hepatic extracts and amniotic fluid as well as with crystalline pepsin but were minimally affected by human urine. The method of isobollic dosages was applied in the determination of secretin activity after various periods of incubation, the ratio of amounts of extract-saline solution to the body fluid mixture, each giving the same pancreatic response in dogs, being a measure of the residual hormonal activity. Some of the cruder products (SI) at 1.0 mg/ml canine serum, displayed inhibition prior to inactivation and the inhibitory phase was diminished or absent with decreasing hormonal concentrations per unit volume of serum. Destruction of secretin activity occurred from the start with SI at 1.0 mg/ml rat serum as was also the case with more highly fractionated types treated with canine serum at the same level but an inhibitory phase was observed with the latter concentrates incubated with horse serum. Although cholecystokinin metabolism paralleled that of secretin in several runs, generally, the rate of inactivation was greater for secretin.

The presence of an enzyme, secretinase, in blood and tissues has been postulated as the causative factor in the *in vitro* inactivation of secretin and in the rapid metabolism of the hormone following intravenous administration (Greengard *et al.*, 1941a). Proteolysis was ruled out on the basis of the relative absence of proteolytic activity in blood serum and in the finding at that time that the hormonal concentrates were not affected by crystalline pepsin and trypsin. Secretinase activity was also demonstrated in urine (Greengard *et al.*, 1941b). Doubilet (1946) regarded the enzyme as similar to choline esterase on the basis of its inhibition by certain drugs and vitamin K. Inactivation of secretin activity by citrated blood plasma in phosphate buffer was likewise observed by Rogers (1951) as was the case with crystalline pepsin and chloroform-activated globulin or serum protease preparation; the cleavage of amino acids did not occur. In this conjunction, the study of Agren and Hammersten (1937) is of interest. Aminipolypeptidase caused the liberation of ten amino acids from a secretin product without any loss in activity. An enzymatic mechanism has also been postulated for the inactivation of cholecystokinin (CCK) by blood serum (Greengard *et al.*, 1941c).

In this study, the method of isobollic dosages advanced by Gershbein *et al.* (1949) was employed for an effective quantitation of the metabolic action of body fluids, tissue extracts and enzymes on secretin and

CCK concentrates. In contrast to the rather qualitative results deduced to date, the present methods allowed for good followup over protracted periods of time and the data could be applied to kinetic evaluations, among others.

Although very potent concentrates are available and in fact, a pure peptide with secretin activity has been synthesized (Bodanszky *et al.*, 1967), the current study with various types of preparations was motivated more by metabolic considerations in relation to 'non-active' moieties associated with or occurring concurrently with secretin in the upper intestinal extracts and which may greatly influence the mode of inactivation as suggested in this report.

Materials and Methods

The secretin concentrates were isolated from the salted acid upper intestinal product of the pig, the A-precipitate, by extraction with 80% ethanol, removal of the alcohol from the filtered solution under vacuum, isoelectric precipitation and heating. The activity was precipitated with trichloroacetic acid to yield the SI, the latter then being converted to the VII-type by treatment with amines in aqueous acetone solution (Gershbein, 1968; Gershbein and Denton, 1967). The acetone-dried A-precipitate (DA) was also used in conjunction with ethanol up to 95% and the activity (ADA) removed by the addition of excess acetone. The ADA was enriched by various treatment schemes, including a picric acid step, the latter giving rise to the P-concentrate (Gershbein and Krup, 1952). In a few runs, clinical samples of secretin (Vitrum) prepared by the methods of the Jorpes' group were also included. The concentrates screened in this study ranged from 0.5 to 300 u/mg for secretin and the CCK extracts contained up to 50 u/mg.

Blood was collected from dogs by cannulation of the jugular and pooled and from horses at the abattoir; rat blood was removed by cardiac puncture. Canine amniotic fluid and blood were pooled during the last quarter of pregnancy. Extracts of dog liver and spleen were prepared by homogenization of the tissue with saline followed by centrifugation of the blend. Spinal fluid was secured from dogs by spinal puncture. Crystalline pepsin (Armour) and a lyophilized concentrate of hog kidney diamine oxidase (DO; Winthrop) containing 1.0 u/mg were employed in the metabolic experiments.

For the evaluation of secretin inactivation by the method of isobollic dosages, a solution of the secretin concentrate in serum or tissue extract, generally at 1.0 mg/ml was incubated at 37°C. together with the control containing an equivalent of the same product in saline. At definite intervals, an aliquot of the serum mixture was removed and injected intravenously into the pentobarbitalized dog with the main pancreatic duct cannulated and the submaximal response (drops of juice) over a period of 10 min ascertained; blood pressure was followed by carotid cannulation. When basal conditions obtained, the animal was standardized against the incubated saline solution. The ratio of dosages of control to serum-treated concentrate, each giving the same response was determined and represented the secretin activity remaining in the incubated mixture. Especially for longer observation periods, asepsis was applied to the bleeding, removal of the serum, preparation of the solutions and to the incubation. The comparisons were carried out against the same saline standard throughout and with a similar mode of handling and where smaller volumes were instituted for rechecking or amplification of values, the corresponding saline control was included at that time. At least two dogs were used in each of the runs.

The metabolism of CCK was also followed by an analogous method and often in the same dog with the pancreatic preparation. The cystic duct was ligated and a metal trocar connected to a glass tube as manometer, secured in the dome of the gall bladder (Gershbein *et al.*, 1949). The corresponding ratio of dosages yielding an equivalent submaximal contractility of the viscus in mm was determined and served as a measure of the residual CCK activity in the incubated mixture.

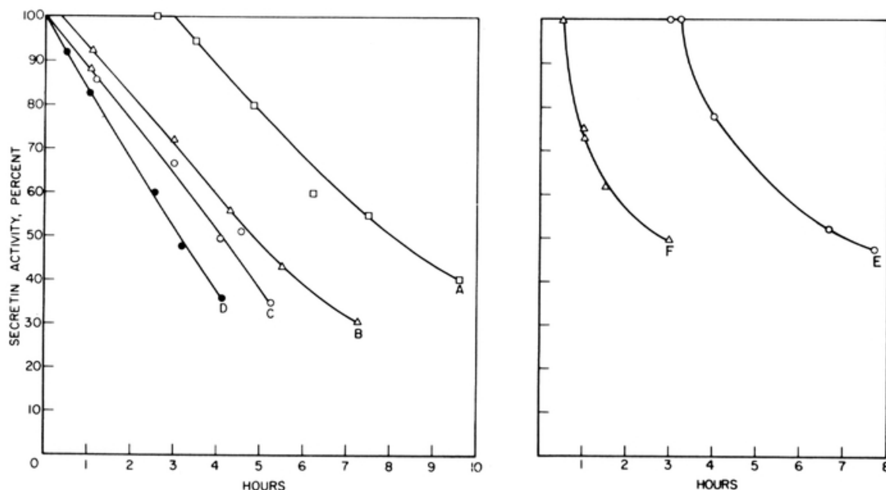

FIGURE 1. Curves depicting the loss of secretin activity on incubation of SI-6 (0.65 u/mg) solutions containing 1.0 mg concentrate in A, 1.0, B, 5 and C, 10 ml of canine serum pool 91 as compared to curve D, SI-6 at 1.0 mg/ml rat serum. E and F were obtained with SI-3 (1.0 u/mg) and SI-4 (4.0 u/mg), respectively, at 1.0 mg/ml canine serum pool 71.

RESULTS

Inactivation of Secretin by Canine Blood Serum

An inhibitory period of about 3 hr was generally noted on incubation of SI concentrates, assaying 0.5-12.5 u/mg, with canine serum at about 1.0 mg/ml before inactivation of the hormone proceeded as exemplified by SI-6 (0.65 u/mg) in Figure 1. The inhibitory period was considerably decreased with a concentration of 1.0 mg per 5 ml of pool 91 (0.13 u/ml) as compared to the 1.0 mg/ml level and essentially absent with the higher dilution by serum (0.065 u/ml); the extent of secretin metabolism increased with diminishing levels of hormonal extract per unit volume of serum (curves A, B and C). SI-3 and SI-4, assaying for 1.0 and 4.0 u/mg, respectively, were also incubated with pool 91 at 1.0 mg/ml and whereas SI-3 simulated the above, SI-4 underwent greater inactivation after a very short inhibitory period (curves E and F). The latter was completely absent on treatment of SI-4 with a second pool but at 1.0 mg/4 ml or 1.0 u/ml (curve C; Figure 2).

The incubation of VII-type concentrates at 1.0 mg/ml serum caused rapid destruction of the hormone without any inhibitory phase even with secretin assaying down to 0.20 u/mg. Typical data are presented in Table 1 for two of the products and illustrated in curves A and B (Figure 2) for a further pool (No. 58) in conjunction with VII-L and VII-R, assaying for 2.0 and 12.5 u/mg, respectively, at 1.0 mg/ml. VII-D (10 u/mg) in addition to the picric acid-fractionated types derived from ADA, P-2 (24 u/mg) and P-10 (50 u/mg) at 1.0 mg/ml and the last product also tested at a level of 1.0 mg per 3 ml of serum pool 82, yield-

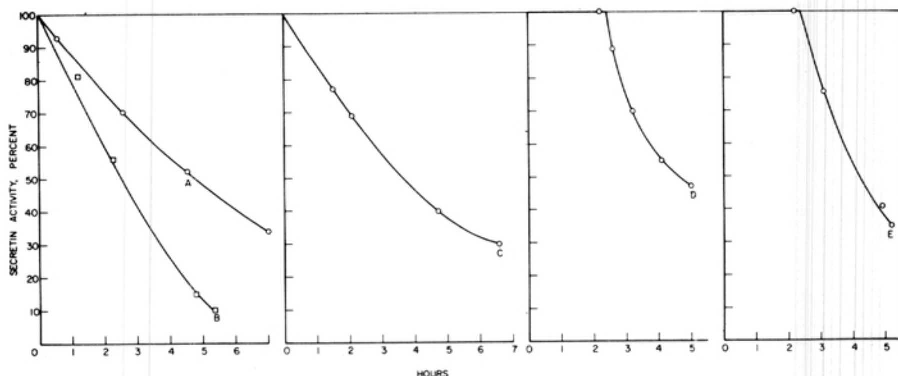

FIGURE 2. Incubation of concentrates with canine body fluids and showing secretin losses with A, VII-L (2.0 u/mg) and B, VII-R (12.5 u/mg) with serum pool 58; C, SI-4 + serum pool 94; D, SI-6 + late pregnancy serum and E, SI-6 with amniotic fluid. In each case, the concentrate has 1.0 mg/ml except for SI-4 at 0.25 mg or 1.0 u/ml.

TABLE 1.—Inactivation of Secretin Concentrates by Canine Serum Pool 70 (1.0 mg/ml).

Incubation Time, min	% Residual Secretin Activity
VII-K (Assay: 0.67 u/mg)	
60	86
153	72
270	52
390	39
VII-R (Assay: 12.5 u/mg)	
60	81
139	56
285	15
323	10

ed the results as advanced graphically in Figure 3. In this regard, the findings with the Vitrum samples, employed up to 300 u/ml, simulated the latter curve, over 80% of the activity being destroyed in 2 hr.

Molecular oxygen was not required for the inactivation of secretin. Thus, the activity remaining 4.25 hr after incubation of SI-4 in serum (1.0 mg/ml) in sealed evacuated tubes was 34% as comparted to 37% after 4.2 hr with the same system in the presence of air.

In all instances, the injection of the incubated sera as well as the tissue extracts and enzyme solutions without hormone at the maximal volumes employed in the respective runs or even higher did not alter the basal pancreatic secretion.

Canine Pregnancy Serum and Amniotic Fluid

The course of inactivation of SI-6 at 1.0 mg/ml of serum pooled from two dogs in late pregnancy as well as of amniotic fluid from such animals, is shown in curves D and E of Figure 2. The inhibitory periods were shorter in either case as compared to sera from nongravid dogs. The incubation of P-2 with the above serum at the same level, led to a loss of 15% of the activity by the end of the first hour and far under 50% after 2.3 hr, whereas, a similar mixture prepared from non-pregnant female serum showed a loss of 27% at the latter period.

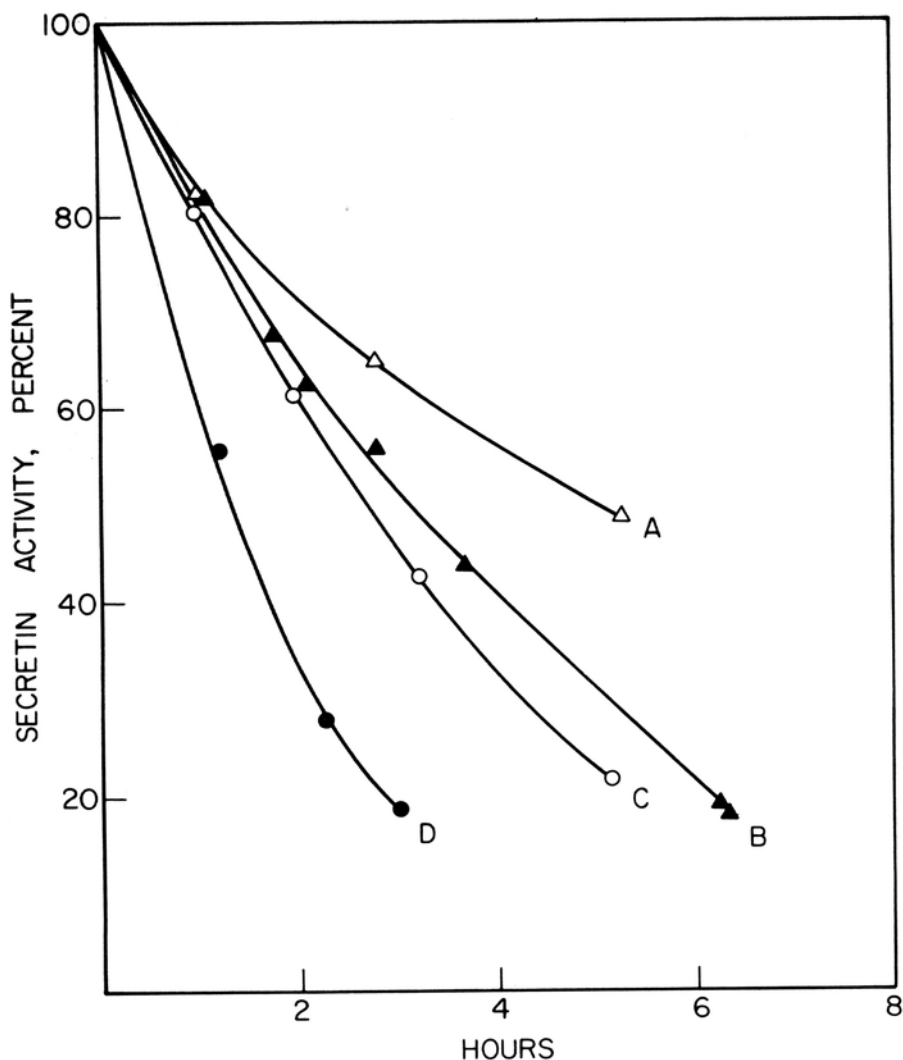

FIGURE 3. Loss of secretin activity with time on incubation of concentrates with canine serum pool 82. A, VII-D (10 u/mg), B, P-2 (24 u/mg) and C, P-10 (50 u/mg) at 1.0 mg/ml serum and D, the last concentrate at 1.0 mg/3 ml serum.

Rat Serum

With serum pooled from rats of either sex, the extent of inactivation of secretin in SI-6 at 1.0 mg/ml exceeded that of the same concentrate or of SI-4 incubated at 1.0 mg/ml and 1.0 mg/4 ml, respectively and no inhibition was observed (curve D; Figure 1).

Horse Serum

Secretin concentrates were treated with serum pooled from mares, stal-

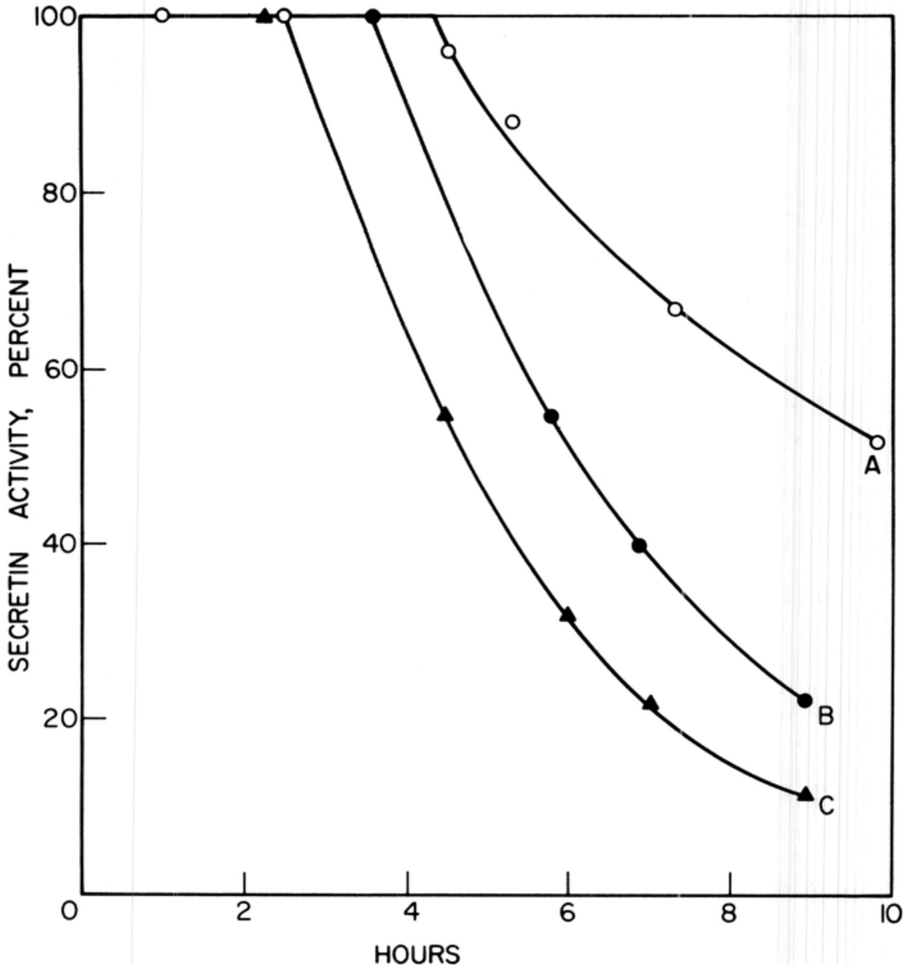

FIGURE. 4. Treatment of concentrates at 1.0 mg/ml horse serum. A, VII-L and pool 10; B and C, VII-R incubated with pools 10 and 12, respectively.

lions and geldings, representative data of which are presented graphically for VII-L and VII-R in Figure 4. The first extract was incubated with a pool from three mares and the second one, with the latter as well as an additional pool, all at 1.0 mg/ml serum. A similar metabolic pattern was obtained with VII-R treated with a pool from four geldings, the secretin activity remaining after 7.3 hr being 67% as was also noted with the mare serum.

Canine Tissue Extracts

On incubation of SI-15 (2.0 u/mg) at 1.0 mg/ml of a 1:80 liver-saline extract, 88% of the hormone was inactivated in 53 min. Under comparable conditions, treatment with 1:300 spleen extract for 50 min caused a loss of 79% and with dilu-

tion of the spleen extract to 1:1,000, 25% was destroyed in 70 min.

Urine and Spinal Fluid

Little metabolism of secretin occurred on incubation of various concentrates with a pool of normal human urine at 1.0 mg/ml and the injection of the mixtures or of the urine alone, generally affected the condition of the assay animal and its standardization. A possible loss of 20% in activity was noted after incubation of 1.0 mg VII-N (9.1 u/mg) in 5 ml of urine for 8.5 hr. Canine spinal fluid of normal or low protein content was essentially without effect on several potent extracts following incubation for 2 hr.

Canine Blood Plasma Fractions

Preliminary screening was carried out with canine plasma fractions I, II, III, IV-1, IV-4 and V isolated by 'method 6' of Cohn *et al.* (1946) and each reconstituted to the respective original level in plasma. Incubation of SI and VII extracts at 1.0 mg/ml of protein solution for 6 hr, led to hormonal losses which were somewhat lower or comparable to those employing the starting fluid or the various serum pools.

Enzymes

Pepsin. - ADA-4 (6.6 u/mg) and P-12 (16 u/mg), each at 1.0 mg per ml of a solution of 1.0 mg% crystalline pepsin in saline, yielded the data depicted graphically in Figure 5 over a period of 1 hr. A similar solution but containing VII-M (9.5 u/mg) underwent somewhat lower metabolism or simulated the behavior of P-12, the residual activities at 35 and 65 min being 42 and 18%, respectively.

DO. - Of several concentrates screened, SI-11 (6.0 u/mg) at 1.0 mg/ml of a saline solution containing 1.0 u DO and P-12, introduced in mixtures with DO at 0.10, 0.50, 1.0 and 2.5 u/ml, underwent losses in hormonal activity, a short inhibitory period occurring with the 0.10 u-level (Figure 6).

Anomalous Behavior Among Animals in Assay of Secretin Concentrates

By the usual *in vivo* assay procedure for secretin (Gershbein *et al.*, 1949), SI, VII and ADA, among other types, yielded virtually identical potency ratios or values from one dog to another as compared to SI or VII standard extracts. However, with about 10-15% of the standardized animals, the assay values were almost half of the expected one with various concentrates, especially those in which a picric acid treatment step was instituted in their fractionation. This, P-4 assayed for 9.0 u/mg in each of 11 dogs but repeated assay produced a value of 4.5 in one animal and 5.0 u/mg, in yet another.

CCK Inactivation

CCK which was quite prominent in SI and VII, in fact, the latter displaying a 1:1 secretin-CCK correspondence in relation to SI (Gershbein, 1968), was followed during the incubation with serum, tissue extracts and enzymes. With many of the SI and VII concentrates, the metabolism of CCK paralleled that of secretin, notably with the higher serum dilutions but usually the rate of inactivation of CCK appeared to be much slower. With SI-4 at 1.0

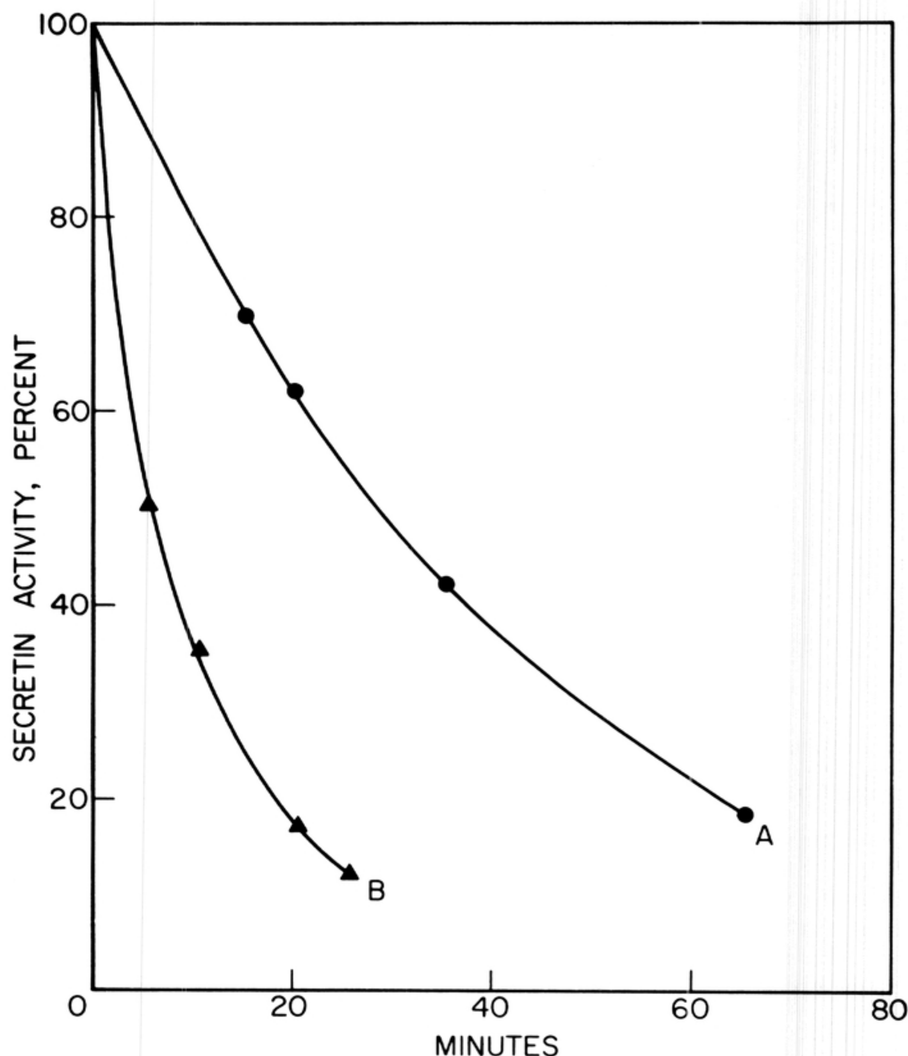

FIGURE 5. A, P-12 (16 u/mg) and B, ADA-4 (6.6 u/mg), each incubated at 1.0 mg/ml of a 1.0 mg% solution of crystalline pepsin in saline.

mg per 5 ml of canine serum at 2.5 hr, both secretin and CCK underwent losses of 31% and a correlation also obtained with SI-6 treated with rat or canine serum. However, incubation of VII-N with dog serum at 1.0 mg/ml for 6.5 hr led to losses of 71 and 29% in secretin and CCK, respectively; in the case of VII-L at a comparable level with horse sera, the residual secretin and CCK activities were 48% each at 9.8 hr. The extent of inactivation of secretin by the DO concentrate also appeared to exceed that of CCK. With SI-11 at 1 mg/u DO, the secretin and CCK

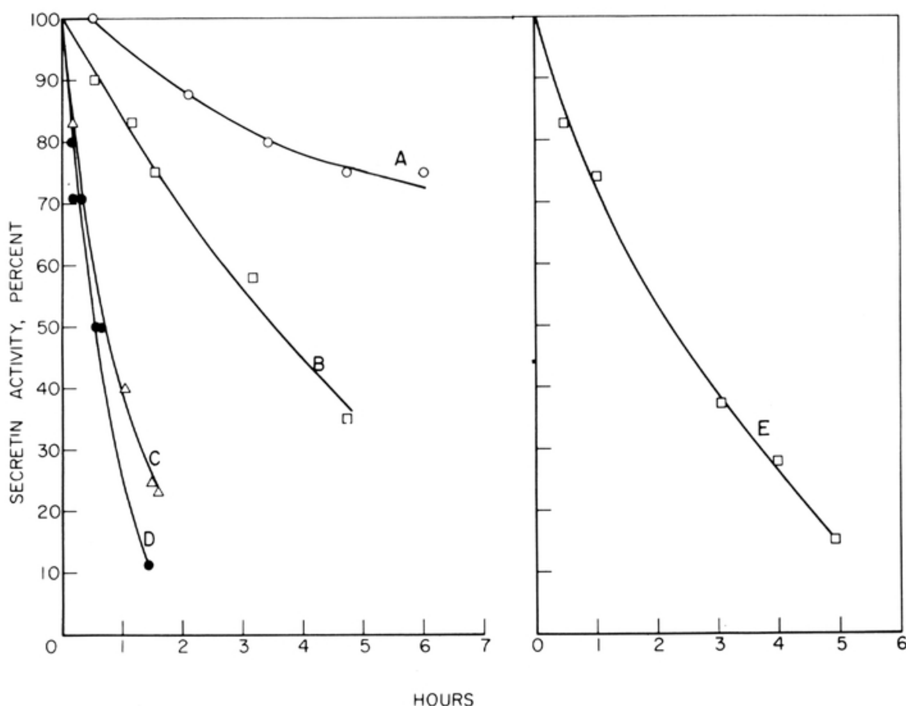

FIGURE 6. Metabolism of P-12 at 1.0 mg/ml of saline -DO solutions containing A, 0.10, B, 0.50, C, 1.0 and D, 2.5 u per ml; E, SI-11 (6.0 u/mg) incubated at 1.0 mg/u DO.

contents at 4.9 hr were 20 and 65% respectively. In another series employing 0.10 u DO/ml, SI-15 displayed activities of 25 and 75% for secretin and CCK, respectively, 18 hr after the start of incubation.

DISCUSSION

The inactivation of secretin in various concentrates has been followed over protracted periods of incubation at 37°C. with serum pooled from such species as the dog, horse and rat. With SI products assaying 0.5-12.5 u/mg, a rather constant finding was the initial inhibitory period occurring at levels of 1.0 mg/ml canine serum and which decreased in duration when the dosage per unit serum volume was diminished (Figure 1): Although the inhibitory phase at 1.0 mg/ml was shorter with SI-4 (4.0 u/mg) and no longer present at 1.0 mg/4 ml as compared to SI-6 (0.65 u/mg), this did not appear to stem solely from the elevated initial unitage. With VII-type extracts as well as ADA and its derived concentrates, destruction of secretin occurred from the start and progressed at rates commensurate with the initial activity or unit concentration per ml of canine serum.

The inhibitory interval noted in the inactivation of SI may originate from preliminary binding with serum protein components prior to pos-

sible enzymatic attack of the transitional product or complex. In yet another approach, it is likely that an enzymatic inhibitor may occur in the preparations, as reflected in the initial phases, considering that VII of equivalent or far lower activity than the SI screened, exhibited no inhibition with canine serum. Also, a definite species difference was evident as regards the observed SI metabolism, under comparable conditions, rat serum causing secretin destruction from the start. However, inhibitors might occur in the serum as well as in the concentrates or in both, considering that VII displayed long inhibitory periods, up to 4-6 hr, on incubation with horse serum (Figure 4).

In the consideration of the effect as due to an enzyme, its concentration in serum may vary in extremes between horse and rat, canine serum ranging intermediately. The postulation of a unique or discrete enzyme, secretinase, rather than of a protease, by Greengard *et al.* (1941a), might not be justified on the basis of their experiments, according to which serum was purported to lack proteolytic acitvity and crystalline pepsin and trypsin, to be without influence on the hormone. Such findings are in marked contradistinction to those of Rogers (1951), among others. Also, early studies had demonstrated that gastric juice rapidly destroys secretin (Carlson *et al.,* 1916). The inactivation by trypsin was attributed by the Greengard group to the alkalinity of the incubated test mixture as such. The enzymatic approach was amplified in the present investigation. Crystalline pepsin was found to destroy secretin activity and in fact, the make-up of the concentrate influenced the action. In this regard, the metabolism of the picrate-purified products appeared to be anomalous in relation to the assay of secretin, 2-3 out of 20 dogs yielding ratios in terms of SI or VII standards of about half of the anticipated value. Accordingly, the type and arrangement of 'inert' components have a great effect on the metabolic properties of the extracts. Such considerations also entered into the efficacy of various schemes in the fractionation of secretin concentrates discussed earlier (Gershbein and Krup, 1952).

The inactivation of secretin by blood serum proceeds in the absence of oxygen and likewise occurs with the plasma protein fractions obtained by the Cohn methods. Canine pregnancy serum appeared to inactivate the hormone more rapidly than nongravid female serum and amniotic fluid was also effective. In contrast to the latter, the destruction of hormonal activity by human urine was minimal.

Although loss of secretin activity was noted with moderate to high levels of the DO product, further purification will be required to pinpoint the metabolic efficacy of the enzyme and to rule out others, notably cathepsins, before significance can be attached to these findings. Additional experiments are necessary to ascertain whether the rapid metabolism of secretin extracts engendered by dilute hepatic and splenic homogenates is on the same or on a different basis compared to blood serum. Although CCK destruction correlated with that of secretin in several instances employing concentrates con-

taining both principles, the extent of inactivation of CCK was generally of a lower order.

The mechanism of secretin metabolism by body fluids and tissue extracts is still open and the role of inhibitors residing in the hormonal concentrates, in the fluid or possibly, in both, is yet to be delineated. With the ease and efficacy of the assay procedures in the screening of incubated mixtures, such secretin studies may provide an attractive model in following the metabolism of proteinaceous hormones of other endocrine systems. The isolation of a pure heptacosapeptide, notwithstanding, the *in vivo* metabolism of secretin must involve preliminary binding with subsequent breakdown of higher molecular weight complexes, discrete units recognized by the body in hormonal metabolic pathways. An investigation of the binding of the heptacosapeptide with blood proteins and tissue extracts would be very timely.

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