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Evidence for Peptide Aggregation

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KASTIN, A. J., P. F. CASTELLANOS, A. J. FISCHMAN, J. K. PROFFITT AND M. V. GRAF. *Evidence for peptide aggregation.* PHARMACOL BIOCHEM BEHAV **21**(6) 969–973, 1984.—Evidence is presented that peptides may occur in aggregated form. Addition of ^{125}I-Tyr-DSIP to serum resulted in four peaks after gel filtration chromatography on a column of Sephadex G-25. One of the peaks (C) eluted at the same position as the labeled peptide standard. Two Peaks (A and B) eluted before the standard and one (peak D) afterwards. The first peak (A) eluted at void volume, a position expected for labeled peptide bound to protein. The other two peaks (B and D), corresponding to smaller molecular size material, were greatly reduced by addition of glacial acetic acid or the chelating agent 1,10-phenanthroline before or even after mixing of the peptide with serum. Iron was one of the ions found to interact with ^{125}I-Tyr-DSIP, and chromatography of a mixture of ferric chloride and peptide without serum resulted in the additional formation of peak B. A substantial portion of peaks A, B, and C (but not D) reacted with a specific DSIP antibody, indicating the presence of intact peptide. The results are consistent with the concept that peptides may occur in multiple forms.

Serum DSIP Protein binding Degradation Precursors Aggregation Phenanthroline

IT is generally assumed that peptides circulate in a "free," unassociated form. Using delta sleep-inducing peptide (DSIP) as a model, we had found that in addition to a free form [5], this nonapeptide also exists in larger forms in the blood of several species including humans [1,6], human cerebrospinal fluid [1], and various rat organs [4]. For somatostatin [8], cholecystokinin [2], gastric inhibitory polypeptide [3], and enkephalin [9], as well as DSIP [1,6], some of these larger forms seem to represent peptide bound to protein. In addition to protein binding, higher molecular weight forms are usually considered to represent precursors (pro-hormones), non-specific interfering substances, or new peptide sequences cross-reacting with an existing antibody. The observations that form the basis of this report indicate that DSIP, and probably other peptides, also can exist in blood in an aggregated form.

EXPERIMENT 1: EFFECT OF MIXING TIME ON THE INTERACTION OF ^{125}I-TYR-DSIP AND HUMAN SERUM

Method

^{125}I-Tyr-DSIP (50,000 cpm) was added to 500 μl of pooled human serum and either immediately chromatogramed or mixed for 15 min or 5 hr at 4°C on a turning wheel (120 rpm). Gel filtration chromatography on a column (1.0×60 cm) of Sephadex G-25 (fine) was performed with each sample as well as with ^{125}I-Tyr-DSIP in 500 μl of column eluent. This eluent consisted of 0.02 M acetic acid containing 0.1% bovine serum albumin (BSA) and 0.002% sodium azide. The flow rate was 20 ml/hr and 2 ml fractions were collected and counted in a gamma counter (Micromedic 4/200). Void volume (Vo) in this and the subsequent experiments was at fraction 10. The radioactivity in each fraction was expressed as a percentage of the total.

The 0 and 15 min mixings were repeated with the addition of 200 μl of distilled water as a control for later experiments involving addition of 200 μl of other substances. The 2 ml fractions from each peak collected after gel filtration on a column of Sephadex G-25 were counted as before and then lyophilized, incubated for 4 days at 4°C with 500 μl of DSIP antibody number 604 (1:1000 initial dilution), mixed at 4°C with charcoal-dextran (0.5%:0.05%), and centrifuged at 1000 g for 30 min. The supernatant and sediment were counted separately in a gamma counter for 5 min and the results expressed as the percentage of bound to total.

Results

Chromatography of ^{125}I-Tyr-DSIP without added serum resulted in a single peak (C) shown in the top panel of Fig. 1. Addition of ^{125}I-Tyr-DSIP to undiluted serum without added water immediately before application to the column resulted in four peaks, as shown in the second panel of Fig. 1. Peak C eluted at the position of the standard labeled peptide. After 15 min mixing, peak B decreased in size and peak D increased (Fig. 1, third panel). This change was more pronounced when the mixing time was extended to 5 hr (Fig. 1, bottom panel).

Figure 2 shows similar results after the 0 min (top) and 15 min (bottom) peptide-serum mixtures (with 200 μl of water added) were chromatogramed on the column of Sephadex G-25. The numbers above each peak represent the percentage of radioactivity bound to antibody added after the chromatography. For both the 0 and 15 min mixing times, about the same proportion of peaks A, B, and C represented material that was bound by the antibody as was found for the mixture without any serum. Very little of peak D was immunoreactive.

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EXPERIMENT 2: EFFECT OF ACIDIFICATION ON THE INTERACTION OF ^{125}I-TYR-DSIP WITH HUMAN SERUM

Method

^{125}I-Tyr-DSIP (50,000 cpm) was mixed with 500 μl human serum at 4°C for 15 min as in Experiment 1 except that 200 μl glacial acetic acid was added either before or after the mixing. Both mixtures were then applied to the column of Sephadex G-25. ^{125}I-Tyr-DSIP added in column eluent without serum was also chromatogramed with and without the addition of the glacial acetic acid as controls.

Results

The top panel of Fig. 3 shows the elution pattern of ^{125}I-Tyr-DSIP without added serum. The addition of acetic acid before chromatography shifted the peak (C) only slightly to the right. Addition of 200 μl of acetic acid to the mixture of ^{125}I-Tyr-DSIP and serum before (Fig. 3, middle panel) and after (Fig. 3, bottom panel) 15 min of mixing resulted in chromatographic patterns similar to those found when the serum was omitted (Fig. 3, top panel). Peaks B and D were no longer present as the distinct peaks seen when water was added (Fig. 2) as a control for the acid. About half the radioactivity of Peak C was immunoreactive in serum as it was in the control without serum, indicating the presence of intact ^{125}I-Tyr-DSIP. The amount of immunoreactive material in the peak increased in proportion to the increase in radioactivity.

EXPERIMENT 3: EFFECT of 1,10-PHENANTHROLINE ON THE INTERACTION OF ^{125}I-TYR-DSIP WITH HUMAN SERUM

Method

This experiment was identical to Experiment 2 except that 200 μl of a 200 mM aqueous solution of the chelating agent 1,10-phenanthroline monohydrochloride monohydrate was added instead of glacial acetic acid. ^{125}I-Tyr-DSIP was applied to the column in eluent alone, in eluent mixed with 1,10-phenanthroline, after mixing with serum and 1,10-phenanthroline for 15 min, and after mixing with serum for 15 min followed by addition of 1,10-phenanthroline. All mixtures were immediately applied to a column of Sephadex G-25.

Results

The top panel of Fig. 4 shows the elution pattern of ^{125}I-Tyr-DSIP applied to the column in eluent alone and with phenanthroline. The addition of 1,10-phenanthroline to the mixture of labeled peptide and column eluent resulted in a shift of the peak to the left. By contrast with controls in which 200 μl of water was added (Fig.2), or even omitted (Fig. 1), addition of 1,10-phenanthroline before (Fig. 4, middle panel) or after the 15 min mixing (Fig. 4, bottom panel) resulted in a chromatographic pattern in which peaks B and D had essentially disappeared. About half the radioactivity of the increased peak C remained immunoreactive, as in the control, even when the phenanthroline was added after the mixing.

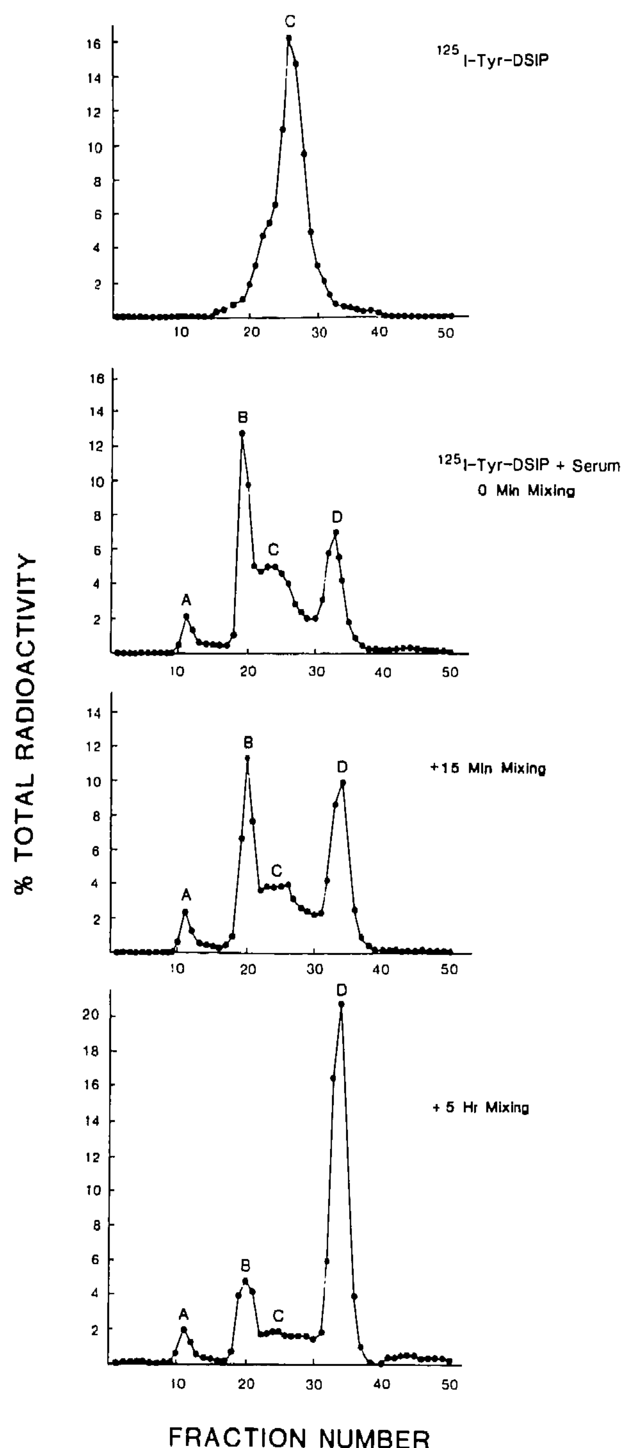

FIG. 1. Gel filtration chromatography on Sephadex G-25 of ^{125}I-Tyr-DSIP (50,000 cpm) in eluent (0.02 M acetic acid, 0.1% BSA, 0.002% sodium azide) alone (top panel), and in human serum (500 μl) after immediate (0) mixing (second panel), 15 min mixing (third panel), and 5 hr mixing (bottom panel). Vo was at fraction 10.

EXPERIMENT 4: EFFECT OF ADDITION OF IONS ON ^{125}I-TYR-DSIP

Method

^{125}I-Tyr-DSIP (50,000 cpm) was incubated for 30 min at concentrations of 10-5000 μM of $FeCl_3$, $FeCl_2$, $ZnSO_4$, $CrCl_3$, $AlCl_3$, $CaCl_2$, KCl, NaCl, $NaHCO_3$, NaH_2PO_4, $MgCl_2$, $CoCl_2$, $NiCl_2$, $CuAc_2$, and $MnCl_2$. Charcoal-dextran (1%:0.1%) was then added and incubated for another 30 min.

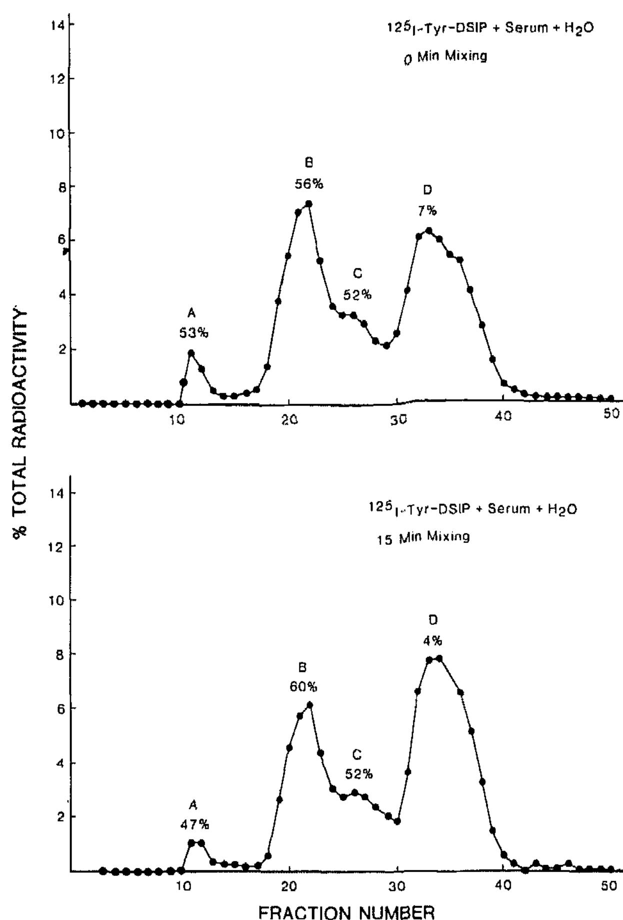

FIG. 2. Gel filtration chromatography of Sephadex G-25 of ^{125}I-Tyr-DSIP in human serum with addition of distilled water (200 μl) as a control at the beginning (time 0; top panel) and end (15 min; bottom panel) of mixing. The numbers above each peak represent the percentage of radioactivity bound after addition of DSIP antibody.

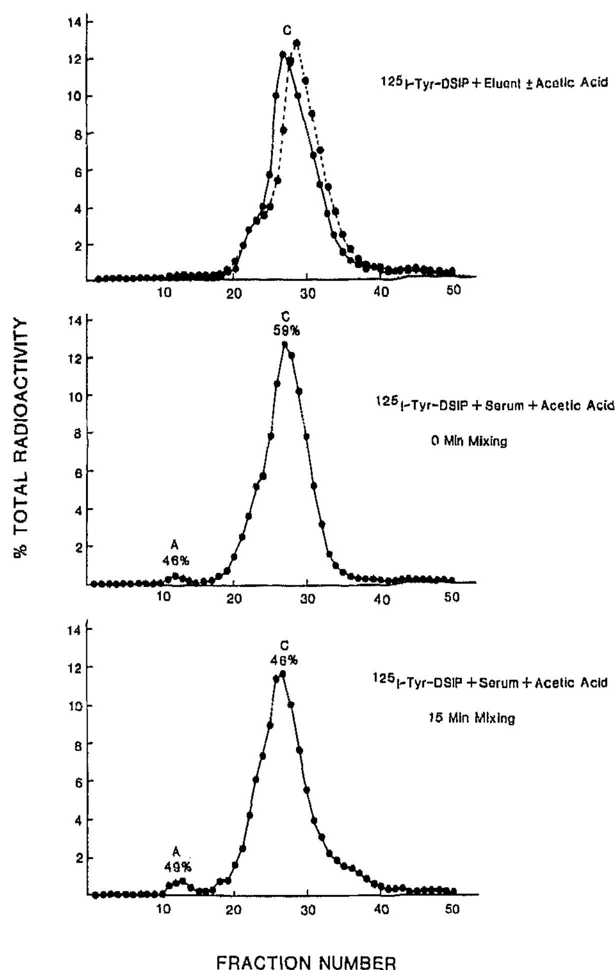

FIG. 3. Gel filtration chromatography on Sephadex G-25 of ^{125}I-Tyr-DSIP in eluent with (dotted line) and without (solid line) the addition of 200 μl glacial acetic acid (top panel). The elution pattern of the mixture of labeled peptide and human serum is shown after addition of 200 μl glacial acetic acid before (middle panel) and after (bottom panel) mixing for 15 min. The numbers above each peak represent the percentage of radioactivity bound after addition of DSIP antibody.

After centrifugation, the supernatant and sediment were counted and the percentage of ^{125}I-Tyr-DSIP not adsorbed by the charcoal-dextran mixture was calculated.

In another part of this experiment, $FeCl_3$ was added at doses of 1.0 and 2.0 ng in distilled water to 50,000 cpm of ^{125}I-Tyr-DSIP. After mixing for 15 min without serum, chromatography on a column of Sephadex G-25 was performed as in the previous experiments.

Results

Several of the ions mixed with ^{125}I-Tyr-DSIP prevented the adsorption of the peptide by charcoal-dextran. $FeCl_3$ exerted the strongest interference, followed by $FeCl_2$, $ZnSO_4$, $CrCl_3$, and $AlCl_3$. No interaction was found after addition of $CaCl_2$, KCl, NaCl, $NaHCO_3$, NaH_2PO_4, $MgCl_2$, $CaCl_2$, $NiCl_2$, $CuAc_2$, or $MnCl_2$.

When $FeCl_3$ was mixed with ^{125} I-Tyr-DSIP in the absence of serum and chromatogramed on a column of Sephadex G-25, a peak occurred at the position of peak B in addition to the usual peak of radioactivity at position C. The percentage of immunoreactivity in peak B, however, was less than that in peak C.

DISCUSSION

The results show that when ^{125}I-Tyr-DSIP was added to normal human serum, three peaks of radioactivity, in addition to the one corresponding to the added peptide occurred after gel filtration chromatography on Sephadex G-25. Possible explanations of the appearance of these peaks include degradation, protein binding, and aggregation. The rapid formation of an iodinated precursor molecule is extremely unlikely. These possibilities will be discussed for each peak.

Peak A eluted at Vo when chromatographed on a column of Sephadex G-25 (Figs. 1 and 2) but was retained on a column of Sephadex G-50. Since the exclusion limit of Sephadex

G-25 is 5,000 and that of Sephadex G-50 is 30,000, peak A can be considered to represent material between these approximate sizes, probably closer to the larger size judging from its position after chromatography on Sephadex G-50. This size range is appropriate for a binding protein. Although in preliminary experiments addition of up to 20 μg unlabeled Tyr-DSIP decreased the radioactivity in peak A by 25–75%, presumably by displacement, the heating of serum at 60°C for 16 hr only decreased peak A by 13%. This is consistent with the possibility that despite its elution position and persistence after treatment with phenanthroline, peak A may represent more than simple protein binding.

For peak A, unlike peaks B and D, incubation of ^{125}I-Tyr-DSIP for 15 min or even 5 hr did not result in an increase in radioactivity (Figs. 1 and 2). This further suggests that a different process was involved in the formation of peak A as compared with peaks B and D. The presence of radioactively labeled peptide in peak A after the immediate addition of the mixture of labeled peptide and serum to the chromatography column effectively rules out the possibility that this peak could represent incorporation of the label by synthesis into a larger molecule such as a pro-hormone or precursor.

The results after addition of DSIP antibody indicate that most of the labeled material in peak A represents the presence of intact ^{125}I-Tyr-DSIP. Of the many fragments previously tested for binding to DSIP antibody number 604, the smallest molecule recognized was desTrp-DSIP (DSIP 2–9), containing all but the first amino acid of DSIP [7]. Since the only peptide added was ^{125}I-Tyr-DSIP, and Tyr is on the N-terminus, desTrp-DSIP would not be radioactively labeled, and a degradation product bound to a large protein would not likely be immunoreactive. The immunoreactivity detected, therefore, should represent intact peptide.

Peak B, like peak A, eluted in a position corresponding to a higher molecular size than the ^{125}I-Tyr-DSIP standard. If peak B represents a protein-bound peptide, the binding protein would have to be much smaller than the exclusion limit of 5000 for Sephadex G-25. This would, of course, apply for protein binding of fragments of ^{125}I-Tyr-DSIP as well as for the intact peptide itself. Moreover, the material in peak B, like the free material in peak C, was bound by the DSIP antibody that does not recognize small fragments of DSIP. With increasing time of incubation, peak B decreased in amount (Fig. 1), whereas the level of bound material would have more likely remained the same (if saturated) or increased.

The production of peak B was prevented by addition of acetic acid and 1,10-phenanthroline (Figs. 3 and 4). The acid could be expected to interfere with protein binding, but this is less likely to explain the effect of 1,10-phenanthroline, a powerful chelating agent. Even more striking, however, were the results obtained with addition of the acid or phenanthroline after the completion of 15 min of mixing ^{125}I-Tyr-DSIP with serum. Under normal experimental conditions (Figs. 1 and 2), a large amount of peak B was formed by this time. Addition of the acid or phenanthroline after the 15 min period during which peak B had already formed resulted in a reversal of this process (Fig. 4, bottom panel) so that most of the radioactivity was shifted back toward peak C, the position of the peptide added in free form as the tracer. Although the glacial acetic acid might affect a protein binding process in this way, the elution position of peak B would necessitate the existence of a binding protein of very small size.

The shift of radioactivity from peaks B and D to the area of peak C when 1,10-phenanthroline was added before

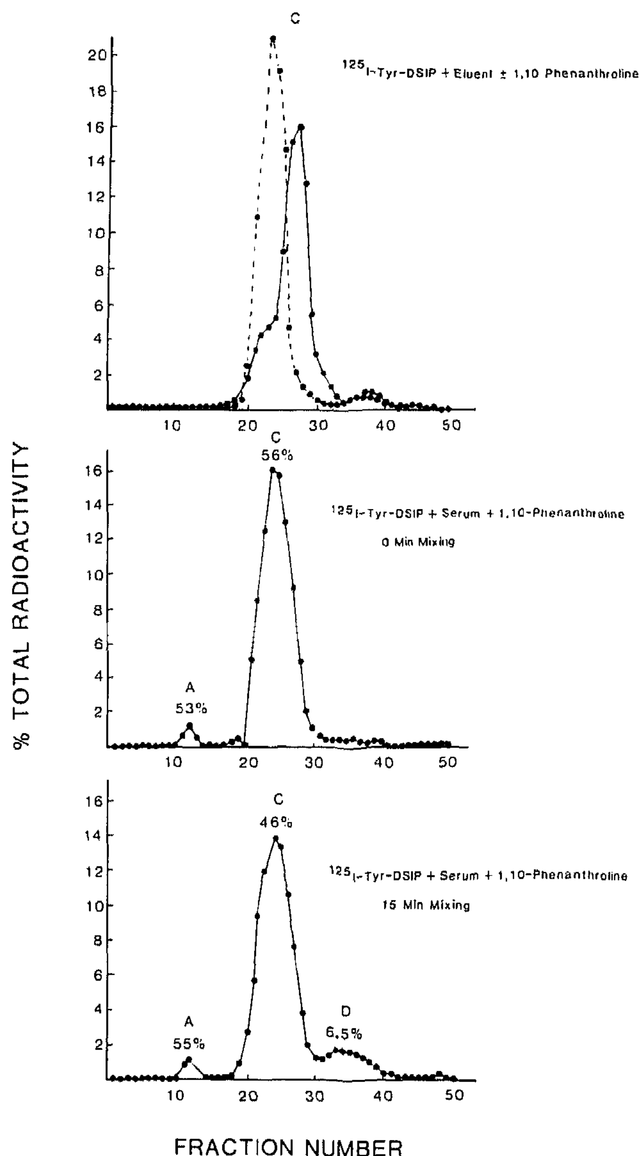

FIG. 4. Gel filtration chromatography on Sephadex G-25 of ^{125}I-Try-DSIP in eluent with (dotted line) and without (solid line) the addition of 200 μl of 200 mM 1,10-phenanthroline (top panel). The elution pattern after chromatography of the mixture of labeled peptide and human serum is shown after addition of 200 μl 1,10-phenanthroline before (middle panel) and after (bottom panel) mixing for 15 min. The numbers above each peak represent the percentage of radioactivity bound after addition of DSIP antibody.

and particularly 15 min after mixing of peptide with serum would be expected if the 1,10-phenanthroline bound an ion that was necessary for self-association of the peptide. The shift of the standard in eluent toward the left by the addition of the phenanthroline was in the opposite direction of the shift from B toward C seen in serum and, therefore, cannot explain the finding. It does suggest, however, the possibility of an interaction of the chelating agent with the labeled peptide that affected its retention on the Sephadex G-25. Addition of serum might have further altered this relationship.

Additional support for aggregation as the explanation for the accumulation of ^{125}I-Tyr-DSIP in peak B was the formation of a peak in the same area when $FeCl_3$ was mixed with ^{125}I-Try-DSIP in the absence of serum. Iron is one of the metallic ions for which 1,10-phenanthroline has high affinity. This is the probable basis for its interference with the apparent aggregation mechanism involving iron. Iron was also the ion that had the greatest effect on the prevention of the removal of ^{125}I-Tyr-DSIP by charcoal-dextran, although zinc, chromium, and aluminum also showed some interaction.

Peak C represents the position at which ^{125}I-Tyr-DSIP eluted when added to a column of Sephadex G-25 without serum (Figs. 1, 3 and 4; top panels). Prevention of the formation of peaks B and D by addition of glacial acetic acid or 1,10-phenanthroline before mixing left peak C with immunoreactive material indicative of the presence of mostly intact material (Figs. 3 and 4). The percentage of antibody binding was about the same as in the solution of ^{125}I-Tyr-DSIP added without serum and approximates maximal binding for the system. When peaks B and D apparently reversed toward the position of peak C after treatment of the already mixed serum and peptide with the acid or phenanthroline, the percentage of material recognized by the antibody remained about the same, but increased in total amount since peak C increased in size. This is consistent with liberation of intact material that had been aggregated.

Peak D eluted at a position of smaller molecular size than the intact material. Since this peak increased in size with time of incubation of labeled peptide in serum (Fig. 1), a degradation product might be considered the most likely explanation; ^{125}Tyr did not appear to elute at the position of peak D, but other N-terminal fragments of ^{125}I-Tyr-DSIP were not available for testing. Although addition of glacial acetic acid would be expected to prevent degradation (as well as binding and aggregation), reversal of the formation of peak D by addition of acid after the mixing of peptide and serum for 15 min was completed (Fig. 3, bottom panel) would not be expected if peak D represents only degradation products. The detection of an increased absolute amount of immunoreactivity in peak C after addition of the DSIP antibody under these conditions in which acid was added after the mixing also would not support only degradation. The prevention and reversal of peak D by incubation with acid, however, might have been explained by protein binding if its position of elution did not correspond to such a small molecular size. Since aromatic compounds can be retarded during gel filtration on Sephadex and elute relatively late, an unusual conformation conceivably might still account for binding in position D. Although 1,10-phenanthroline can inhibit enzymatic activity, the apparent ability of the phenanthroline to prevent and reverse the formation of peak D (Fig. 4) constitutes evidence that at least part of peak D may represent an aggregated form of ^{125}I-Tyr-DSIP associated with an ion.

Even though peak D may partially represent an aggregated form of the labeled peptide, this form is probably different from the aggregated form present in peak B. It is conceivable that peak B might represent an aggregated form of ^{125}I-Tyr-DSIP in which the Tyr and Trp side chains are internalized, perhaps around a ferric ion, whereas peak D might represent an aggregated form of the labeled peptide in which these aromatic side chains are externalized and hence greatly retained during chromatography on Sephadex. Further evidence for the differences between these two peaks is the lack of immunoreactivity of peak D in contrast to its presence in peak B.

The process of aggregation of labeled DSIP may also occur with other peptides. Gel filtration of Sephadex G-25 of ^{125}I-Tyr-somatostatin added to serum and mixed for 15 min revealed a new peak eluting earlier (nearer Vo) than when the peptide was added to eluent alone and chromatogramed. With labeled GRF added to serum, an additional peak was also found, but it eluted 5 fractions later after gel filtration chromatography on Sephadex G-25 and thus could have represented a degradation product. This was not found with ^{125}I-LHRH that eluted about 18 fractions earlier when chromatogramed in serum on Sephadex G-25 than when chromatogramed in eluent. When 250 μg $FeCl_3$ were mixed at 4°C with the labeled LHRH without serum, a small additional peak of radioactivity began to emerge after 15 min that became the predominant peak after 24 hr. This peak of radioactivity formed by the mixture of ^{125}I-LHRH and $FeCl_3$ in eluent alone eluted at the same position as the peak of ^{125}I-LHRH mixed without iron in serum.

Thus, taken together, the evidence indicates that aggregation of peptides may occur in blood. The forms of aggregation probably differ, and different ions may be involved. The implications of this finding for the actions of peptides and their penetration into organs like the brain remain to be established.

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