[CONTRIBUTION FROM THE DEPARTMENT OF CLINICAL SCIENCE, UNIVERSITY OF ILLINOIS COLLEGE OF MEDICINE]

Secretin Studies. I. The Preparation of Potent Secretin Concentrates[1]

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Procedures have been developed toward the isolation of potent secretin concentrates. By dehydration with acetone, salted acid pig intestinal extracts have been converted into stable powders (DA) which serve as excellent starting materials, being low in bile, fat and other contaminants. A study has been made of the extraction of the hormone from DA by means of alcohol solutions and the properties of the resulting products (ADA) have been investigated. Trichloroacetic acid converts ADA into more active fractions, whose potency can be further augmented by treatment with aniline. The addition of picric acid to such products or to the ADA directly, generally gives rise to highly active concentrates. A preliminary molecular weight value and the amino acid content have been determined for the most potent product (100 units of secretin/mg.) obtained in this study. The relationship between the resulting secretin unitage arising from a variety of purification schemes and the starting ADA concentrate is discussed.

Since the discovery of Bayliss and Starling[2] in 1902 that a specific agent elaborated by the upper intestinal mucosa stimulates pancreatic secretion, numerous attempts have been made to isolate the active principle, secretin. Although innumerable concentrates have been reported, their evaluation is ambiguous due to the inadequate assay methods employed. Also, the presence of vasodilatins and coexisting impurities makes comparison of their activities very difficult. Thus, the product of Mellanby[3] thought to be pure secretin, was shown to contain vasodilatins and hypoglycemic moieties.[4] The isolation of crystalline secretin derivatives have been claimed by two groups of investigators. Hammarsten, Wilander and Ågren[5] converted a salicylate obtained in an electrodialytic procedure to a slightly soluble crystalline picrolonate after crystallization from pyridine. A similar crystalline product, also employing pyridine as solvent, was isolated by Greengard and Ivy,[6] starting with a salted acid intestinal extract from hogs ("A-precipitate"). Doubt has been cast on the purported crystallinity of the secretin picrolonate through X-ray diffraction studies[7] which show that the pattern of the Greengard and Ivy picrolonate from pyridine is identical with that of pyridine picrolonate.

The characterization of secretin as a polypeptide stems from Mellanby[3] and the Hammarsten group.[5] The latter obtained a molecular weight of about 5000 by the ultracentrifuge method for a secretin phosphate prepared from the picrolonate. The diffusion constant method fixed the value at 1800 for a concentrate purified by way of lecithin.[8] Takacs[9] describes his product as a secondary albumose containing 15% nitrogen, the latter diminishing upon dialysis. Cunningham[10] believes his preparation to be a secondary proteose adsorbable by precipitated proteins from acid and neutral solutions. It could be ultrafiltered through cellophane and retained by peptone-permeable collodion membranes. In contrast to the Greengard and Ivy product,[6] free amino groups occurred in the Hammersten preparation as evidenced by the liberation of 7% of its nitrogen upon treatment with nitrous acid. It is of interest to note that the latter concentrate liberated ten amino acids on digestion with aminopolypeptidase without any loss of activity.[11] The Swedish group believes that these acids are an integral part of the secretin molecule.

In the present study, the assay procedure advanced by Gershbein, Wang and Ivy[12] was employed in the evaluation of secretin activity. In contrast to others, this method, which is relatively rapid and reliable, involves the determination of the ratio of dosages of a standard to unknown, both producing the same pancreatic response in anesthetized dogs. The total nitrogen content did not constitute an index of secretin potency since the respective values for starting and enriched concentrates were comparable.

The "A-precipitate," a salted acid intestinal extract, first described by Weaver, Luckhardt and Koch,[13] has enjoyed widespread use in secretin fractionation procedures. It is putty-like in consistency, contains 60–72% water and around 20% mineral matter. However, fat, bile and vasodilatins are abundant, and rancidity can at times be detected on prolonged storage. These impurities often persist even to the final steps of purification. In order to remove the bulk of water and allow for greater ease in handling, especially as regards solvent extraction, the "A-precipitate" was dried by a number of methods. The one subsequently adopted comprised blending with acetone, wherein a large amount of the contaminants were removed; little or no secretin occurred in the acetone liquors. The resulting yellow-green powders (DA) contain up to 60% inorganic matter, possess good keeping qualities, and serve as excellent starting materials. Some samples which had been stored in the ice-chest for a period of one year showed little diminution in activity as evidenced by subsequent processing. Although duodenal powders have been prepared,[14,15] this is the first instance of a dry, stable

(1) Presented in part before the Biochemical Division of the American Chemical Society, San Francisco, California, April, 1949. This study was aided by grants from The Toni Company, Abbott Laboratories and the National Institutes of Health.

(2) W. M. Bayliss and E. H. Starling, *J. Physiol.*, **28**, 325 (1902).

(3) J. Mellanby, *ibid.*, **66**, 1 (1928).

(4) E. Zunz and J. LaBarre, *Arch. intern. physiol.*, **31**, 162 (1929).

(5) E. Hammarsten, G. Ågren, A. Hammarsten and O. Wilander, *Biochem. Z.*, **264**, 275 (1933).

(6) H. Greengard and A. C. Ivy, *Am. J. Physiol.*, **124**, 427 (1938).

(7) H. Greengard, M. L. Wolfrom and R. K. Ness, *Federation Proc.*, **6**, 115 (1947).

(8) G. Ågren and O. Wilander, *Biochem. Z.*, **259**, 365 (1933).

(9) L. Takacs, *Z. ges. exptl. Med.*, **60**, 424 (1928); *ibid.*, **63**, 553 (1928).

(10) R. N. Cunningham, *Biochem. J.*, **26**, 1083 (1932).

(11) G. Ågren and E. Hammersten, *J. Physiol.*, **70**, 330 (1937).

(12) L. L. Gershbein, C. C. Wang and A. C. Ivy, *Proc. Soc. Exp. Biol. and Med.*, **70**, 516 (1949).

(13) M. M. Weaver, A. B. Luckhardt and F. C. Koch, *J. Am. Med. Assoc.*, **87**, 640 (1926).

(14) J. H. Burn and P. Holton, *J. Physiol.*, **107**, 449 (1948).

(15) W. Stepp, *ibid.*, **43**, 441 (1912).

product which retains the potency of the original acid extract.

In order to ascertain the maximum secretin unitage obtainable from the DA concentrates, a series was blended with successive portions of 95% ethanol and the respective filtrates treated with an excess of acetone yielding assayable white powders (ADA). As can be noted from Table I, over 75% of the extractable activity occurred in the first two fractions. Table II shows results obtained with two successive extractions of DA with ethanol (A and B fractions; 4 ml. of alcohol per gram of DA) in concentrations of 70–100%. Most of the unitage was removed with 80–85% solutions. It must be mentioned that except for some activity obtained with absolute methanol, a few per cent. of water in the solvent is necessary. Thus, absolute ethanol or 2-propanol in direct contrast to the aqueous solutions extracted little or no secretin; 1-butanol failed as a medium, the small amount of solid removed being toxic to the assay animal. No advantage was afforded by the use of either alcohol containing acetone or 70% acidified acetone. With decreasing ethanol concentrations, the unitage of secretin per milligram of ADA fell with concomitant increasing inorganic content. On a dry and ash free basis, the potencies were at times comparable (Table II).

The ADA concentrates are white solids, low in moisture content and moderately soluble in water; traces of acid aid solution. The usual amino acid tests are positive except for the Millon's color reaction which was negative or inconclusive for several fractions. This is also reflected in the ultraviolet absorption spectra as shown in Fig. 1. Often, especially with higher concentrations of extracting alcohols, no maximum was obtained in the region of 275–278 mμ, indicative of aromatic amino acids (curve A, spectrum of ADA-A10-95%A). In many of the enrichment procedures for ADA to be discussed, the spectrum often remained unchanged as illustrated by Curve B, in which the maximum persists even after picric acid treatment. The dialysis of ADA solutions against water using cellophane or Visking casing at 3° for 22 hours gave rise to concentrates low in inorganic matter; some secretin dialyzed through the membranes. Cholecystokinin, the hormone concerned with gall bladder contraction, and closely associated with secretin in the upper intestinal mucosa, occurred in the ADA concentrates. However, it was more abundant in those extracts prepared with lower alcohol concentrations in agreement with earlier reports.[16] Solutions containing 85–95% ethanol were employed in the extraction of DA since cholecystokinin was invariably lower and generally greater enrichment was possible by various procedures. This made recourse to isoelectric precipitation as recommended by others[6] unnecessary.

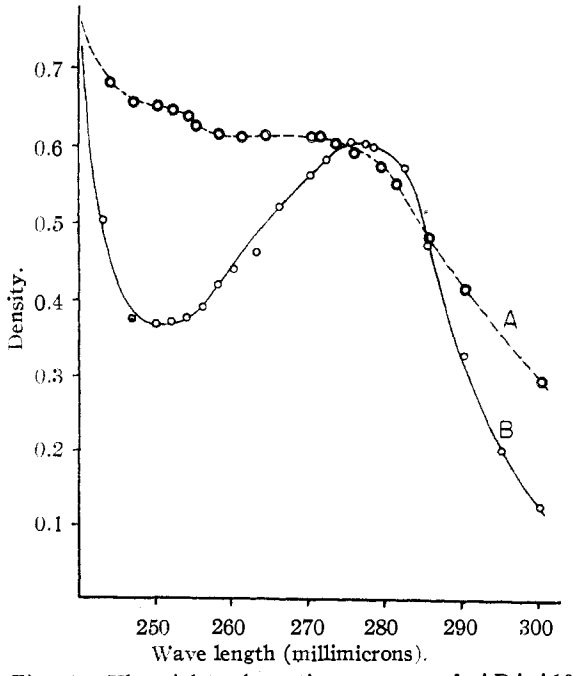


Fig. 1.—Ultraviolet absorption spectra of ADA-A10-95%A (curve A, 1.57 mg./ml.) and P-923-85%A (curve B, 1.00 mg./ml.).

It should be pointed out that the mere removal of inorganic matter does not constitute enrichment, but rather the unequivocal increase in potency as expressed by secretin unitage per milligram on a dry and ash-free basis. The treatment of ADA with 95% ethanol or absolute methanol led to only moderate potency increases; the starting material was recovered on a weight and unitage basis with 1-butanol. Lloyds reagent adsorbed the activity; a variety of exchange resins tested were ineffective. When an aqueous solution of ADA was shaken with chloroform, under 1% of the total occurred in the chloroform–water interface with almost all of the activity recoverable from the aqueous phase.

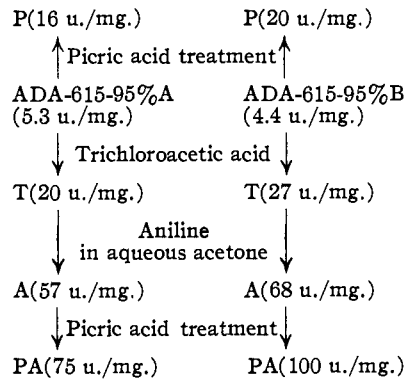
The addition of an aluminum hydroxide suspension to an equal volume of 2.5% aqueous ADA solution led to minor enrichment. Even greater increases resulted from treatment of ADA with trichloroacetic acid (Table III). The secretin content per milligram of resulting precipitate could be further augmented by the addition of amines such as aniline in aqueous acetone solution by a modification of the procedure of Greengard and Ivy.[6]

Another approach to the fractionation of ADA concentrates consisted in the treatment of the aqueous solutions with picric acid (comprising at least three times the weight of ADA). The resulting precipitates on trituration with 70% acidified acetone and treatment of the filtrates with an excess of acetone, yielded products low in inorganic matter, and with an average total nitrogen content of 14.5% (Table III). Methanol containing 1% concentrated HCl by volume yielded comparable and in a few cases somewhat greater activity than with 70% acidified acetone; acetone alone or with small amounts of acid (up to 2% HCl by volume) was less effective. Although several fractions underwent little enrichment with picric acid, except for the removal of inorganic matter, many showed up to fivefold enrichment. Thus, ADA-A10-95%-B (5.25 u./mg. or about 13 u. on a dry and ash-

(16) H. C. Lueth, A. C. Ivy and G. Kloster, *Am. J. Physiol.*, **91**, 329 (1938).

free basis) upon treatment with picric acid gave rise to a fraction assaying for 50 u. of secretin/mg. The behavior of such concentrates toward amino acid test reagents simulated that of the respective ADA fractions but unlike the latter or the corresponding trichloroacetate product, the cholecystokinin content was unequivocally low. In some cases, tannic or picrolonic acids yielded products with comparable or lower unitage than the respective picrate-purified fractions. When a few of the latter were treated with saturated tannic acid solution, less than 5% of the initial unitage occurred in the resulting precipitates.

For the attainment of very potent secretin concentrates, combinations of the above procedures were employed,[17] especially the addition of picric acid to the trichloroacetic acid preparation before or after aniline fractionation. Although the yields on a total unitage basis were often low, the main purpose was the isolation of a product of maximum potency. Thus, the trichloroacetic acid concentrate assaying 22 u./mg., obtained from ADA-923-85%A (3.0 u./mg., Table II), yielded material by way of picric acid containing 50 u./mg. However, even greater activity was achieved as shown in the scheme



The values in parentheses denote the assay results; except for the relatively high inorganic content of the ADA (10–11 u./mg. on a dry and ash-free basis), the ash of the other products ranged under 4%. PA-615-95%B (100 u./mg.; total nitrogen: 14.1%) constitutes the most potent concentrate produced in the present study. No free amino acids occurred in this fraction; the amino acid analysis of the acid hydrolysate by paper chromatography appears in Table IV.

It can be noted that the ADA-95%B yielded products which were consistently higher in potency than those from the ADA-95%A. Such differences in the A and B fractions were repeatedly observed. In fact, preliminary measurements by the monolayer spreading method[18] for PA-615-95%A and Pa-615-95%B place the average molecular weights at 5,000 and 13,500 respectively.[19] Undoubtedly, the nature and distribution of the matrix or moieties onto which the hormone is adsorbed or associated play an important role in subsequent extraction and purification procedures. In this connection, it must be emphasized that the starting product is an extremely complex mixture derived from the pig intestine, and unless care is exercised in obtaining fresh materials and in the preliminary processing, products of low potency can result which may resist further fractionation.

Studies to date with several of the potent products suggest their possible clinical application since vasodilatins are absent and they are nonantigenic to the guinea pig.

(17) Picric acid promoted little enrichment of ADA dialysis residues or aluminum hydroxide filtrates.

(18) H. B. Bull, *J. Biol. Chem.*, **126**, 585 (1938); THIS JOURNAL, **67**, 4 (1945).

(19) Little change in the average molecular weight of A-615-95%B resulted on further treatment with picric acid (Table V).

Experimental

Materials.—Distilled water and C.P. grade reagents were used throughout this study except in the preparation of the "A-precipitate." The following resins or adsorbents were employed: Lloyds reagent (Eli Lilly and Company), Zeo-Karb H, Zeo-Dur, Deacidite-735, Permutits H, Q and S (The Permutit Company) and IR-100 (The Resinous Products and Chemical Company). The aluminum hydroxide suspension was prepared by treatment of a 1% alum solution with 1% ammonium hydroxide at 25°.[20] The gel in amount of 100 g. yielded 0.79 g. of aluminum oxide on ignition.

Common Techniques.—Invariably, centrifugation was employed for the filtration of concentrates. Fractions precipitated with excess acetone were washed with a 1:1 acetone–ether solution and finally ether before drying under vacuum over solid sodium hydroxide. For the moisture content, the loss in weight at 110° was determined. By further heating of the sample in a muffle furnace at 675°, the percentage ash was obtained. Micro-Kjeldahl technique as well as Nesslerization was employed in the evaluation of the total nitrogen content. The ultraviolet absorption spectra were obtained with a Beckman model DU spectrophotometer in the range of 220–320 mμ.

Assay Procedure.—The *in vivo* method described by Gershbein, Wang and Ivy[12] was used for the assay of secretin concentrates. The pancreatic duct of the dog under pentobarbital anesthesia was cannulated, and aqueous solutions of the concentrates injected into the femoral vein. The ratio of dosages of the standard extract (arbitrarily assigned a value of 1.0 unit secretin/mg.) to unknown, each giving the same drop rate over a ten-minute period, was taken as the number of units contained in one milligram of the test product. At least 2–3 animals were employed in the evaluation of each fraction. Cholecystokinin was assayed by determination of a similar ratio of standard to unknown dosages, both causing the same gall bladder contraction in dogs with a cannula secured in the bladder after ligation of the cystic duct.[12]

Acetone Drying of the "A-Precipitate" (DA)

A. Preparation of the "A-Precipitate."—Essentially the procedure as described by Greengard and Ivy[6] was employed for the collection of the "A-precipitate." The first 2–5 feet of intestine was removed from freshly slaughtered pigs, and each was everted, the adhering contents gently freed by hand and the strips allowed to remain in contact with cold 0.4% HCl (200 ml. of acid per intestine) for a period of 20–30 minutes with frequent stirring; batches of 20–50 were handled at one time. The intestines were wrung out, the acid extract filtered through cheese cloth to remove large particles, and the contents saturated with commercial sodium chloride. The resulting "A-precipitate," after filtration through paper, was stored in the ice-chest. The yield per strip ranged from 5–20 g. depending on the size of the intestine. Generally, the time between slaughter and eversion averaged 30–40 minutes. Aside from the inorganic content (around 20%), each "A-precipitate" contained from 60–72% of water depending on the time involved in the filtration of the salted extract.

B. Acetone Drying Procedure.—For the removal of water from the "A-precipitate," drying with acetone was adopted. Each kilogram of the precipitate was blended in a Waring blendor with 4 l. of acetone, the contents were allowed to stand at 25° for a period of about 24 hours after which time the greenish supernatant fluid was decanted.

(20) G. Tracy and W. H. Welker, *J. Biol. Chem.*, **22**, 55 (1915).

Two liters of acetone was then added to the residue with stirring and after 5 hours, the contents were filtered under suction through a buchner funnel; air drying for 24 hours followed. Large pieces present at the start of the drying period were pulverized. The yellow-green powders (DA) which contained under 5% moisture and up to 60% inorganic matter were stored in the ice-chest. The yield of DA averaged one-third of the weight of the starting "A-precipitate." That little or no activity occurred in the discolored acetone liquors was evident from assaying the residues obtained upon vacuum concentration or by further addition of acetone to portions of these solutions. In the preliminary drying experiments, "A-precipitates" of known water content were blended with acetone so that the final concentrations of the latter were 80, 85 and 95%, respectively. Alcohol extraction of these dried powders yielded fractions which showed little or no difference in their secretin content; the specified 4 l. of acetone for the above drying gives a concentration of about 85%. In the present study, 60 collections of "A precipitate," averaging 400 intestinal strips per batch, were converted into DA concentrates by the above drying procedure.

Experiment 54.—The "A precipitate," in amount of 4.4 kg. (obtained from 860 strips), was blended with 17.6 l. of acetone, and after standing for 24 hours, the supernatant solution was decanted and the residue stirred with 8.8 l. of acetone. The yield of DA, after filtration and air-drying was 1.65 kg.

Alcohol Extraction of Dried "A-Precipitates" (ADA)

A. Successive Extractions with 95% Ethanol.—The secretin unitage obtainable from DA was determined by blending several with successive portions of ethanol; sample data employing 95% ethanol appear in Table I. In several runs, the blending of DA by hand with successive smaller portions of 95% ethanol, did not lead to any greater activity on the basis of secretin unitage/mg. of concentrate; however, the total unitage extracted was less.

TABLE I

EXTRACTION OF DA CONCENTRATES WITH SUCCESSIVE PORTIONS OF 95% ETHANOL

Batch[a]	95% EtOH[b] used, ml.	Yield,[c] mg.	Secretin assay, u./mg.	Total secretin unitage	% of total unitage in fraction
DA-100					
A	150	550	3.7	2035	46.5
B	150	560	2.2	1232	28.2
C	100	355	1.5	533	12.1
D	100	290	1.1	319	7.3
E	100	260	0.75	195	4.5
F	100	260	0.25	65	1.5
G	100	260[d]	..	..	..
H	100	260	..	..	..
I	100	260	..	..	..
DA-104					
A	150	575	1.2	690	49.6
B	150	710	0.70	497	35.7
C	100	370[e]	0.55	204	14.7
DA-EI					
A	150	955	1.1	1051	70.7
B	150	700	0.44	308	20.8
C	100	350	0.36	126	8.5

[a] Batches of 50 g. of DA were employed in the three series. [b] The Waring blendor was used in all cases; about 65–80 ml. of alcohol was required for the wetting of the DA concentrate. [c] These ADA fractions showed progressively higher amounts of inorganic matter with successive extractions. [d] Fractions G, H and I contained very small quantities of secretin (under 2% of the total). [e] Fractions D-G inclusive were exceedingly low in secretin activity.

Experiment 50.—A total of 50 g. of DA-100 was blended in a Waring blendor with 150 ml. of 95% ethanol, and the filtrate (80 ml.) was introduced into 800 ml. of acetone and chilled for 16 hours. The precipitate, after filtration and washing with 40 ml. of 1:1 acetone–ether and two 40-ml. portions of ether, weighed 550 mg. Further extraction of the residual DA with alcohol yielded fractions as shown in Table I.

B. Aqueous Ethanol Solutions.—The above study was extended to alcohol concentrations of 70–100%, employing 4 ml. of alcohol solution for each gram of DA concentrate. The latter was re-extracted with the same volume, and the filtrates upon treatment with excess acetone yielded the A and B fractions (ADA). Table II lists pertinent analytical and assay data of representative ADA concentrates obtained by alcohol extraction of DA. Large dosages were required for the assay of the 70 or 75% A and B ADA extracts. Solutions of 95% ethanol containing acetone in amounts up to 20% showed less promise as compared to 95% ethanol. Thus, 25 g. of DA-923 on treatment with a solution of 80 ml. of 95% ethanol and 20 ml. of acetone yielded 190 mg. of ADA assaying 4.8 u./mg. (total unitage, 912) in comparison with 5.2 u./mg. or a total of 1092 u. with 95% ethanol.

Experiment 57. A and B Extraction of DA-923 with 85% Ethanol.—Upon extraction of 25 g. of DA-923 with two 100-ml. portions of 85% ethanol in a Waring blendor, filtrate volumes of 55 and 98 ml., respectively, were obtained. Treatment of each with 1 l. of acetone yielded white powders weighing 670 and 1170 mg. and assaying for 2.5 and 1.25 u./mg., respectively. On an ash- and moisture-free basis the unitage of both are quite similar as can be noted from Table II.

Experiment 137. A and B Extraction of DA-317 with 95% Ethanol.—Similar to the above, two extractions of 1800 g. of DA-317 with 7.2 l. of 95% ethanol gave rise to ADA fractions in amounts of 19 g. of A and 15 g. of B. On an ash- and moisture-free basis, the secretin activities were 19 and 13 u./mg., respectively.

C. Anhydrous Alcoholic Extracts.—That small amounts of water are required in the alcohol extraction of DA can be noted from Table II. Thus, absolute ethanol removed only very small amounts of a brown solid of low potency; similar results were obtained with anhydrous 2-propanol and 1-butanol. Absolute methanol, in marked contrast to the above, definitely extracted activity from the DA but to a lesser degree than 95% ethanol.

Experiment 140. Absolute Methanol Extraction of DA-2W.—Whereas the extraction of 50 g. of DA-2W by 150 ml. of absolute methanol yielded 1070 mg. of ADA (1.3 u./mg.; total unitage 1391), 95% ethanol produced 580 mg. containing 2.8 u./mg. or a total of 1624 units.

Experiment 73. 2-Propanol Extraction of DA.—When 50 g. of DA-A10 was blended with 150 ml. of absolute 2-propanol, no detectable activity occurred in the filtrate. However, with the alcohol containing 20% water, 25 g. of DA-107 yielded 225 mg. (360 u.), definitely lower than the unitage obtained with 80% ethanol.

D. Properties of ADA Concentrates. 1. Amino Acid Tests.—Except for the Millon color reaction which was questionable for some of the concentrates, the usual amino acid tests (ninhydrin, Hopkins–Cole, biuret, Sakaguchi and xanthoproteic) were positive.

2. Ultraviolet Absorption Spectra.—The ultraviolet spectra of a series of ADA prepared by use of aqueous alcohol solutions (70–95%) were determined. Several of the 95% fractions showed a flattening at 275–278 mμ (curve A, Fig. 1) in which region a maximum was generally exhibited by products obtained from treatment of DA with lower concentrations of alcohol (similar to curve B, Fig. 1).

3. Occurrence of Cholecystokinin.—Although cholecystokinin occurred in all ADA concentrates, it was especially prominent in those resulting from the extraction of DA with solutions containing 70–80% ethanol. Thus, ADA-A10-95% A contained 8.25 u. of secretin/mg. and 2.5 u. of cholecystokinin/mg., whereas the corresponding values for ADA-923-80%A were 2.5 u./mg. for each.

4. Chloroform Treatment.—Very little solid or activity occurred in the interface on treatment of aqueous solutions of several ADA fractions with chloroform.

Experiment 82.—One gram of ADA-93-85%A (1.9 u./mg.) in 40 ml. of water when shaken with 8.5 ml. of chloroform led to the separation of 6.4 mg. of an inactive gray solid at the interface.

5. Dialysis.—The behavior of ADA concentrates on dialysis is exemplified by the following experiments with cellophane.

TABLE II

EXTRACTION OF SECRETIN FROM DA CONCENTRATES BY 70–100% ETHANOL CONCENTRATIONS (ADA FRACTIONS)

Concentrate (g.)	EtOH concentration, %[a]	Fraction[b]	Yield,[c] ADA, mg.	Assay values, u./mg.[e] Actual	Corrected	Total secretin unitage	% H_2O	% Ash	Total N ash-free basis
DA-A10 (25)	95[g]	A	190	8.25		1568			
	90	A	350	5.0		1750			
	85	A	806	3.9		3144			
	80	A	856	3.0		2586			
	70	A	1787	1.25		2234			
	95	B	590	5.25		3098			
	90	B	725	2.5		1813			
	85	B	2070	0.75		1553			
	80	B	2350	0.75		1763			
DA-923 (25)	100	A	21[d]	0.55		12			
	90	A	340	4.4	12	1496	5.00	57.72	12.46
	85[f]	A	670	2.5	10	1675	5.16	69.25	12.55
	80	A	950	2.0	11	1900	57.0	76.56	12.76
	90	B	435	2.5	12	653	5.23	74.72	14.06
	85[f]	B	1170	1.25	11	1462	4.19	84.83	11.58
	80	B	1970	0.75	7.4	1478	1.45	88.54	15.50
DA-93 (25)	100	A	16[d]	0.50		8			
	95	A	250	3.1	5.3	775	3.93	37.07	
	90	A	510	2.5	5.7	1275	4.37	51.81	12.55
	85[f]	A	750	2.25	6.0	1688	1.30	61.54	12.11
	80	A	1200	1.25	4.6	1500	0.51	72.60	12.64
	75	A	1301	1.1	5.2	1431	1.18	77.50	
	70	A	1900	0.63	4.8	1197	1.00	85.93	
DA-EI (25)	100	A	42[d]	0.2		84			
	95	A	580	1.2	2.1	696	2.17	40.76	12.62
	90	A	796	1.1	2.0	876	2.80	41.54	10.96
	85	A	1320	0.95	2.1	1244	2.06	53.34	11.66
	80	A	1820	0.83	2.3	1511	1.37	61.93	11.72
	70	A	3250	0.20	0.80	650	1.30	73.67	11.98
DA-317 (500)	85	A	15,000	1.5	4.5	22,500	0.41	66.19	11.55
	85	B	19,400	1.1	7.1	21,340	1.56	83.04	
DA-54 (500)	95	A	5,250	1.8	4.0	9,450	3.00	52.46	15.16
	90	A	9,200	1.0	2.7	9,200	2.99	60.54	13.81
	85	A	20,000	0.82	3.5	16,400	1.48	75.24	
	95	B	4,630	1.6	3.6	7,410	2.85	52.79	13.50
	90	B	23,700	0.50	2.5	11,850	1.29	79.01	13.09
	85	B	38,000	0.44	3.5	17,720	1.21	86.07	
DA-615 (1100)	95	A	12,500	5.3	9.8	66,250	3.88	41.88	13.34
	95	B	12,000	4.4	11	52,800	2.56	57.38	11.88

[a] 4 ml. of the ethanol solutions was employed per gram of DA. [b] A refers to the first extraction of DA with a given alcohol solution; where the residue is again treated with the ethanol, this is designated by B. [c] The filtrate after centrifugation and treatment with at least a ten volume excess of acetone, yielded these ADA concentrates. [d] These fractions were brown-tan in color. [e] An actual assay value signifies secretin unitage contained in one milligram of the concentrate; the corrected figures refer to unitage on a dry and ash-free basis. [f] Minor differences in secretin content occurred on processing larger batches of DA(500 g.); such extracts were included in subsequent enrichment studies. [g] For the ultraviolet absorption spectrum, see Fig. 1.

Experiment 98.—A solution of 1.00 g. of ADA-93-85%A (1.9 u./mg. or 5.1 u. on a dry and ash-free basis) in 40 ml. of water contained in a cellophane sac was dialyzed against 150 ml. of water at 4° for 22 hours. The residue retained by the membrane was introduced into 600 ml. of acetone. The product, 214 mg., contained 5.6 u./mg. Similar results were obtained with Visking casing.

Experiment 139.—When 703 mg. of ADA-317-95%A (6.6 u./mg.; 19 u. on a dry and ash-free basis) in 40 ml. of water was dialyzed in cellophane against 200 ml. of water for 72 hours at 3°, the yield of residue after processing as in the last experiment was 166 mg., containing 10 u./mg. (11 u. on a dry- and ash-free basis).

6. **Treatment with Adsorbents.**—When aqueous solutions of ADA were treated with a variety of adsorbents by the batch or column method and the respective filtrates introduced into excess acetone, little or no increase in activity resulted; these comprised IR-100, Zeo-Karb H, Zeo-Rex, Deacidite-735, Permutits H, Q and S and Zeo-Dur. Definite losses in activity occurred with Permutit S and Deacidite-735; Lloyds reagent adsorbed almost all of the hormone activity. Only a small enrichment was observed by the use of an aluminum hydroxide suspension.

Experiment 138. Aluminum Hydroxide Suspension.—A solution of 7.0 g. of ADA-317-95%B (assay: 4.4 u./mg. or 13 u. on a dry and ash-free basis) in 275 ml. of water was stirred with 275 ml. of aluminum hydroxide suspension for five minutes. The contents were filtered by centrifugation, the filtrate (300 ml.) treated with 3.5 l. of acetone and chilled for 16 hours. The product, which weighed 412 mg., assayed 14 u./mg. on a dry and ash-free basis.

Experiment 80. Lloyds Reagent.—A total of 2.5 g. of Lloyds reagent was added to 40 ml. of a 2.5% aqueous solution of ADA-93-95%A. After two hours at 25° with frequent shaking, the contents were filtered and the resulting filtrate introduced into 600 ml. of acetone. The solid (330 mg.) contained less than 0.1 u./mg.

7. **Alcohol Extraction.**—The trituration of powdered ADA with absolute methanol or 95% ethanol led to concentrates of reduced inorganic content with moderate or no

TABLE III

TREATMENT OF REPRESENTATIVE BATCHES OF ADA WITH TRICHLOROACETIC AND PICRIC ACIDS

	ADA				T-Concentrate[b]			P-Concentrate[d]		
Batch	Weight, g.	Volume of H_2O, ml.	Assay values, u./mg.[a] Actual	Corr.	Yield, mg.	Assay values, u./mg.[a] Actual	Corr.	Yield, mg.	Assay values, u./mg.[a] Actual	Corr.[e]
A45-95%A	5.00	300	1.0	2.0	1170	4.1	4.3			
A315-85%A	2.00	40	0.55	2.2	148	4.0	5.5			
	0.503	45	0.55	2.2				46	5.3	5.5
54-95%A	2.00	80	1.8	4.0	231	13	14			
	1.00	45	1.8	4.0				164	5.3	5.9
54-95%B	2.00	80	1.6	3.6	244	16	17			
	1.00	40	1.6	3.6				211	12	12
54-90%A	5.00	1000	1.0	2.7	430	6.4	7.1			
54-85%A	15.0	700	0.82	3.5[c]	1500	4.0	4.6[c]			
	1.00	40	0.82	3.5				25	4.0	4.5
54-85%B	20.0	1000	0.44	3.5[c]	1500	5.1	5.8			
615-95%A	10.0	500	5.3	9.8	1910[g]	20	22			
	0.500	40	5.3	9.8				115	16	17
615-95%B	10.0	600	4.4	11	1515[g]	27	29			
	0.500	40	4.4	11				96	20	21
714-85%A	7.50	500	3.2	10	1265	12	14			
714-85%B	17.5	975	1.0	6.3	1100	13	15			
317-95%A	0.105	50	6.6	19	15	20	22			
	17.0	900	6.6	19				3500	16	16
	0.900	60	6.6	19				165	16	16
317-95%B	3.00	150	4.4	13	452	26	29			
	1.00	40	4.4	13				98	32	34
A10-95%B	0.319	16	5.25	12				31	50	53
A315-95%A	0.502	30	2.2	3.6				27	8.0	8.4
A315-90%A	0.507	30	2.0	4.0				73	8.0	8.4
A315-85%B	1.00	45	0.55	1.7				48	4.0	4.2
32-95%A	3.00	800	1.5	3.0				180	12	12
36-95%A	3.00	800	1.2	2.4				135	9.0	9.2
923-85%A	1.00	55	3.0	10				121[f]	22	23
923-85%B	1.00	55	1.5	11				52	8.0	10

[a] *Cf.* note *e*, Table II. [b] Trichloroacetic acid (6.1 *N*) was added to the respective ADA solutions to a final concentration of 5%. [c] Estimated values as based on averages of ash and moisture contents obtained from a series of similar fractions. [d] An average of 7 g. of picric acid in 5 ml. of warm acetone (under 40°) was added to each gram of ADA in solution; the precipitate (per 0.5–1.0 g. of ADA) was triturated with 30–45 ml. of 70% acidified acetone before treatment with excess acetone. [e] Since the ash contents of P-concentrates were invariably under 1%, the corrected assay values are essentially on a moisture-free basis; the total nitrogen values averaged 14.50%. [f] For the ultraviolet absorption spectrum, see Fig. 1. [g] Further fractionation of such products by way of aniline are exemplified by Experiments 156b and 157b.

enrichment; 1-butanol extracted little or no activity from these fractions.

Experiment 149.—When 7.00 g. of ADA-317-85%A (1.5 u./mg. or 4.5 u. on a dry and ash-free basis) was triturated with 175 ml. of 95% ethanol, a total of 1.40 g. of product resulted (moisture, 5.38%; ash, 14.85%; assay, 4.7 u./mg. or 5.7 u. on a dry and ash-free basis).

Enrichment of Concentrates with Trichloroacetic and Picric Acids

A. Trichloroacetic Acid.—The method of Greengard and Ivy[6] was used in the preparation of trichloroacetic acid products. The acid (6.1 *N*) was added to the aqueous ADA solutions so as to give a final concentration of 5%. Table III lists a number of products obtained in this way. Further enrichment of these fractions was possible by way of amines such as aniline in aqueous acetone solution by modification of the Greengard and Ivy scheme. The procedures are illustrated by the following:

Experiment 156a. Preparation of the T-Concentrate from ADA-615-95%A.—To 10.0 g. of ADA-615-95%A (assay: 5.3 u./mg. or 9.8 u. on a dry and ash-free basis) in 600 ml. of water, was added 35 ml. of 6.1 *N* trichloroacetic acid with stirring. After 3 days in the ice-chest, the contents were filtered by centrifugation and dried with acetone–ether solutions of increasing ether concentration and finally ether. The concentrate, 1.91 g. (assay: 20 u./mg.), analyzed for 6.91% moisture and 1.31% ash.

Experiment 156b. aniline Treatment of T-615-95%A.—An amount of 1.50 g. of T-615-95%A was triturated with 30 ml. of water containing 0.1 ml. of concentrated HCl until solution occurred. A total of 120 ml. of acetone followed by 3.7 ml. of freshly distilled aniline, was added with stirring, and the liquid decanted from the precipitate after filtration by centrifugation. Two additional treatments of the residue (weight after drying, 640 mg.; 0.95 u./mg.) were carried out in the same manner. The combined supernatant fluids on chilling for two hours deposited a solid (132 mg.; 3.5 u./mg.). The filtrate was rid of acetone and aniline under vacuum and at a temperature below 45°; several additions of water were required for the complete removal of aniline. The aqueous solution together with washings, totalling 20 ml., was filtered from a tan-colored solid (3.4 mg.; assay, 2.4 u./mg.), treated with 100 ml. of acetone and chilled for 24 hours. The resulting tacky solid (A-615-95%A), after drying in the usual manner, weighed 271 mg. (assay, 57 u./mg).

Experiment 157a. Preparation of the T-Concentrate from ADA-615-95%B.—A sample of 10.0 g. of ADA-615-95%B (assay: 4.4 u./mg. or 11 u. on a dry and ash-free basis) on treatment with trichloroacetic acid according to the above procedure, yielded 1.52 g. of concentrate containing 27 u./mg. (ash, 3.91%; moisture, 2.92%).

Experiment 157b. Aniline Treatment of T-615-95%B.—Similar to Experiment 156b, 1.10 g. of T-615-95%B yielded 174 mg. of product (A-615-95%B); assay, 68 u./mg.

The T-concentrates appeared to be somewhat less water-soluble than the corresponding ADA fractions; the ash contents ranged under 7%. Aniline treatment led to highly water-soluble and virtually ash-free fractions. Cholecystokinin was present in these products in varying amounts depending on the ADA employed.

B. Picric Acid.—The addition of picric acid in acetone to aqueous solutions of ADA, followed by trituration of the resulting precipitates with 70% acidified acetone (1 ml. of concentrated HCl in 9 ml. water and 400 ml. acetone),[21] often led to the separation of potent concentrates upon addition of excess acetone (Table III).

Experiment 141.—A solution of 500 mg. of ADA-615-95%B (4.4 u./mg. or 11 u. on a dry and ash-free basis) in 40 ml. of water was treated with 7 g. of picric acid in 5 ml. of acetone. The precipitate was triturated with 45 ml. of 70% acidified acetone, the filtrate added to 500 ml. of acetone and chilled for 16 hours; yield 96 mg. (20 u./mg.).

Although the amount of picric acid employed constituted 3–7 times the weight of ADA, larger additions did not appear to affect the secretin content. When picric acid precipitates were triturated with acetone or with the latter containing 0.5–2% concentrated HCl by volume, the resulting concentrates were less potent than those obtained with 70% acidified acetone or methanol–1% HCl as media.

Experiments 105–110.—Six picrate precipitates, each formed by the addition of 7 g. of picric acid in 5 ml. of acetone to a solution of 1.00 g. of ADA-923–85%A (3.0 u./mg. or 10 u. on a dry and ash-free basis) in 55 ml. of water, were triturated with 25-ml. portions of the following solutions: acetone, acetone containing 0.5, 1 and 2% concentrated HCl by volume, 70% acidified acetone and methanol–1% HCl. Their respective yields and assays are as follows: 224 mg. (11.5 u./mg.); 194 mg. (11.5 u./mg.); 194 mg. (11.5 u./mg.); 225 mg. (11.5 u./mg.); 121 mg. (20 u./mg.) and 77.9 mg. (24 u./mg.).

Although tannic and picrolonic acids enriched several of the ADA fractions used, the final products were often not as potent as those employing picric acid, the procedure which was finally adopted. Tannic acid in some cases gave rise to products that were gummy and not so easily handled.

Experiments 125–129.—Whereas ADA-293-85%A yielded a concentrate containing 20 u./mg. (*cf.* Experiments 105–110), the same ADA (500 mg. in 20 ml. of water) formed a precipitate with a saturated aqueous tannic acid solution which on processing as in the above rise to 86.5 mg. (12 u./mg.). In another run employing a slurry of 2.0 g. of picrolonic acid (Eastman Kodak Co.) in 20 ml. of acetone, 1.00 g. of the above ADA formed a product which after analogous treatment assayed for 14 u. on a dry and ash-free basis.

In some cases, the concentrates from tannic and picric acid treatment were similar in unitage. Thus, with 1.00 g. of ADA-923-85%B (0.75 u./mg. or 7.0 u. on a dry and ash-free basis) yields and assays of the resulting relatively ash-free concentrates were 46 mg. (8.6 u.) and 52 mg. (8.0 u.) with tannic and picric acids, respectively.

The effects of tannic and trichloroacetic acid on picrate-treated fractions are illustrated in Experiments 173 and 174.

Experiment 173.—A saturated solution of tannic acid was added to 510 mg. of P-317-95%A (16 u./mg.) in 40 ml. of water to complete precipitation. The contents were filtered, and the precipitate on processing with 70% acidified acetone and excess acetone, yielded 85 mg. of product (4.0 u./mg.).

Experiment 174.—To a solution of 204 mg. of P-317-95%A (16 u./mg.) in 100 ml. of water was added 5.5 ml. of 6.1 *N* trichloroacetic acid. After chilling for 2 days, the precipitate was washed and dried in the usual manner; yield 105 mg. (18 u./mg.).

The following runs exemplify results obtained by the addition of picric acid to the T- or A-concentrate.

Experiment 201.—To 72.4 mg. of T-923-85%A (22 u./mg.) in 4 ml. of water was added 510 mg. of picric acid in 0.5 ml. of acetone. The filtrate obtained on triturating the precipitate with 2.0 ml. of 70% acidified acetone and treatment with 25 ml. of acetone, gave rise to 11.4 mg. of a white solid containing 50 u./mg.

(21) The 70% acidified acetone medium was not as effective as alcohol solutions in the extraction of secretin from the DA products.

Experiment 165.—Similar to the above, the picrate precipitate from 54 mg. of A-615-95%A (56 u./mg., Experiment 156b) in 30 ml. of water produced 21 mg. (PA-615-95%A; some loss) containing 75 u./mg.

Experiment 211.—In exactly the same manner, 48.9 mg. of A-615-95%B (68 u./mg., Experiment 157b) yielded 20.0 mg. of product (PA-615-95%B) assaying 100 u./mg. after picric acid treatment (nitrogen content, 14.10%).

The nitrogen content and amino acid tests for the water-soluble concentrates obtained by way of picric acid simulated those of the T- and A-concentrates. The relative cholecystokinin content was definitely lower than that of the respective ADA or T-extracts. Thus, the assay of PA-615-95%B (100 u. secretin/mg.) showed cholecystokinin to occur in amounts far under 3.0 u./mg.

Amino Acid Composition of PA-615-95%B (100 u./mg.).—PA-615-95%B was hydrolyzed by heating with 20% HCl in a sealed tube for 17.5 hours, after which time the tube was opened, and the contents vacuum dried. Groups of amino acids were determined by paper chromatography.[22] Free amino acids were absent in the original sample as evidenced by paper chromatography of solutions containing larger amounts of concentrate.

TABLE IV

AMINO ACID COMPOSITION OF PA-615-95%B (100 U./MG.)

Area	Amino acids	Per cent.
1	Aspartic acid, cysteine, and salt effect[a]	4.98
2	Lysine	9.13
3	Glutamic acid, serine and glycine	29.43
4	Arginine and threonine	3.72
5	Alanine and tyrosine	14.74
6	.....	2.32
7	Valine	5.98
8	Phenylalanine, methionine, leucine and isoleucine	26.30
9	.....	3.42

[a] The salt effect originated from the tiny amount of inorganic matter present (under 1%).

Molecular Weight Determinations.—By the monolayer spreading method, preliminary molecular weight data were obtained for three of the potent concentrates as shown in Table V; spreading was carried out on 5% ammonium sulfate and 2% glycerol.

TABLE V

MOLECULAR WEIGHT DATA OBTAINED BY MONOLAYER SPREAD METHOD

Concentrate	Secretin, units/mg.	Molecular weight
PA-615-95%A	75	5,000
A-615-95%B	68	12,000
PA-615-95%B	100	12,500
		14,500

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(22) H. B. Bull, J. W. Hahn and V. H. Baptist, THIS JOURNAL, 71, 550 (1949).