[CONTRIBUTION FROM THE LABORATORIES OF THE ROCKEFELLER INSTITITE FOR MEDICAL RESEARCH]

The Occurrence of the Growth Factor Strepogenin in Purified Proteins[1]

BY HERBERT SPRINCE[2] AND D. W. WOOLLEY[3]

The bacterial growth factor, tentatively called strepogenin, which stimulates the growth of a variety of bacteria, has been found in partial hydrolysates of vitamin-free casein.[4] It has therefore seemed advisable to examine several purified proteins for their ability to yield the factor on partial hydrolysis. Since Wright and Skeggs[5] have reported strepogenin-like activity for enzymic digests of casein, we have tested such preparations and found them to be more active than the partial hydrolysates prepared with dilute acid as described from this Laboratory.[4] Therefore, proteins to be examined were treated with trypsin rather than with acid.

Tryptic digests of many proteins were found to be much richer sources of the growth factor than was the liver extract previously used as a starting point for purification. Most of these proteins were highly purified, and had been recrystallized repeatedly. All proteins, however, did not yield strepogenin activity when trypsinized. For example, dialyzed egg white, a crude proteinaceous material, proved to be almost inactive, while crystalline proteins such as insulin and trypsinogen gave rise to activity far greater than that of liver extracts.

The rate of liberation of strepogenin by the action of trypsin varied with different proteins. Maximal activity was obtained with digestion periods of twenty hours or less. In the case of casein, more strepogenin was released by trypsin than by pepsin.

Since tryptic digests of certain proteins were richer in the growth factor than sources previously examined, these digests have been used as starting points for the purification of strepogenin. Several schemes for concentration of the substance have been tested, and a few will be outlined in the experimental section. Some properties of the active agent which shed light on its chemical nature also will be included.

Experimental

Methods.—The strepogenin assays were performed with the aid of *Lactobacillus casei* according to the method described earlier.[4] It is necessary to emphasize, as indicated previously, that a small inoculum was used in this test, since with larger seedings the entire effect may be less well marked. In the preparation of the basal medium, it was found advisable to use the casein hydrolysates made with sulfuric acid as recommended rather than the hydrochloric acid hydrolysates commercially available. In the case of the sulfuric acid procedure, traces of strepogenin which survived the acid hydrolysis were removed by the barium sulfate,[6] which was used to rid the solution of sulfate ion. When commercial hydrochloric acid hydrolysates were employed, the growth in the blank tubes was appreciable, but acceptable assays could nevertheless be obtained. A protein to be examined was dissolved or dispersed in water and heated at 100° for three minutes. The suspension was cooled, adjusted to *p*H 7.6–8.0, made to a concentration of 5 mg. per cc., and treated with an amount of purified trypsin (Fairchild) equal to 1% of the weight of the protein. The *p*H was maintained by small additions of alkali and the mixture was incubated at 37° under toluene. The routine time of incubation was twenty hours since, as will be illustrated below, no more activity was liberated from several proteins on longer digestion. The reaction mixture was then acidified to *p*H 3, heated to 100°, cooled, neutralized, centrifuged if necessary, and assayed.

Sources of Proteins.—The crystalline trypsinogen, three times recrystallized trypsin, three times recrystallized chymotrypsins, seven times recrystallized chymotrypsinogen, four times recrystallized ribonuclease, the crystalline yeast proteins, and the crystalline trypsin inhibitor were kindly supplied by Dr. M. Kunitz of The Rockefeller Institute for Medical Research. The crystalline insulin was supplied by Dr. G. B. Walden of Eli Lilly Company. The salmine, hemoglobin, gelatin, and vitamin-free casein, were commercial samples. Dr. W. M. Stanley of The Rockefeller Institute for Medical Research supplied the crystalline tobacco mosaic virus. The egg white was prepared by heating fresh egg white, grinding, suspending in water, dialyzing against running water, filtering and drying. Digests of undialyzed egg white showed activity approximately equal to that of the liver standard in the assay.

TABLE I

RELATIVE STREPOGENIN CONTENTS OF PROTEINACEOUS MATERIALS DIGESTED WITH TRYPSIN FOR TWENTY HOURS

Material assayed	Relative strepogenin content
Liver fraction L	1
Dried beef pancreas	0.5
Crystalline trypsinogen	30
Crystalline trypsin	13
Commercial trypsin (Fairchild)	10
Crystalline chymotrypsinogen	2
Crystalline chymotrypsin	16
Crystalline gamma chymotrypsin	9
Crystalline ribonuclease	10
Crystalline insulin	40
Crystalline trypsin inhibitor[a]	0
Crystalline yeast protein 2	6
Crystalline yeast protein 3	3
Yeast yellow protein	1
Crystalline tobacco mosaic virus	6
Casein (vitamin-free)	5
Hemoglobin	6
Dialyzed egg white	less than 0.1
Gelatine	less than 0.1
Salmine	less than 0.1

[a] Because it was impossible to digest trypsin inhibitor with trypsin, the assay was done on an intact sample which had been heated at 120° in water at *p*H 6.8 for fifteen minutes.

(1) An abstract of this paper was published: Woolley and Sprince, *Federation Proceedings*, **4**, 164 (1945).

(2) Fellow of The Nutrition Foundation, Inc.

(3) With the technical assistance of B. Bailey and M. L. Collyer.

(4) Sprince and Woolley, *J. Exp. Med.*, **80**, 213 (1944).

(5) Wright and Skeggs, *J. Bact.*, **48**, 117 (1944).

(6) Woolley, *J. Exp. Med.*, **73**, 487 (1941).

Strepogenin Content of Various Proteins.—In Table I the relative strepogenin contents of various proteins as determined by *L. casei* on twenty-hour tryptic digests are shown. The standard of reference for activity was Wilson's liver fraction L which was assigned a potency of 1. The sample of liver fraction used in this work gave half-maximal effect usually at a level of 70 gamma per cc. of culture. The values in Table I may not be a true measure of the strepogenin content of the materials examined. They represent only the activity for *L. casei* liberated from various proteins under comparable conditions of tryptic digestion. It may be that other means of hydrolysis would yield higher values.

Rate of Liberation of Strepogenin Activity from Various Proteins.—Strepogenin activity was not liberated by trypsin from various proteins at the same rate. The data in Table II show that for all proteins examined, maximal liberation had occurred at or before twenty hours.

TABLE II

RATE OF LIBERATION OF STREPOGENIN ACTIVITY[a] FROM PROTEINS BY TRYPSIN

Proteins	Relative strepogenin activity liberated by trypsin in 2 hr.	3 hr.	6 hr.	20 hr.	48 hr.
Crystalline trypsin	13	..	..	13	..
Crystalline trypsinogen	30	..	..	30	..
Crystalline chymotrypsin	4	..	..	16	..
Crystalline ribonuclease	..	..	10	10	12
Crystalline insulin	..	..	11	40	33
Casein	..	..	3	5	5
Hemoglobin	..	2	..	6	5

[a] The values are expressed relative to the liver fraction L which was assigned a potency of 1.

Release of Strepogenin Activity by Pepsin.—Pepsin digestion of casein at *p*H 1.8 under the conditions used for the experiments with trypsin gave rise to only slight activity. Moreover, the maximal growth obtainable with peptic digests was not as great as with tryptic digests or as with the liver extract standard. Therefore, the response differed qualitatively as well as quantitatively from that normally observed. When the incubation of casein with pepsin was followed by digestion with trypsin the same value was found for strepogenin activity as was observed for tryptic digests alone. Therefore, it was concluded that the action of pepsin did not destroy the potentiality of the protein for yielding strepogenin.

Concentration of Strepogenin from Casein Digest. (a) By Differential Extraction with Phenol.—One hundred grams of casein (Labco vitamin-free) was dissolved in 1 liter of water by the addition of sodium hydroxide to a final *p*H of 8. One gram of purified trypsin was added, and the mixture was incubated at 37° under toluene for four hours. The solution was acidified to *p*H 4.5 with hydrochloric acid, heated to boiling, cooled, and poured into a closed cellophane tube. The material was next dialyzed against 4 changes of 6 liters of water. Each period of dialysis was at least six hours in length before the water was changed. The combined dialysates were evaporated at low temperature to about 300 cc., the resulting suspension was adjusted to *p*H 6, cooled to 4°, and filtered. The filtrate was extracted three times with 300-cc. portions of phenol. The phenol extracts were then combined and extracted twice with 100 cc. of water plus 10 cc. of concentrated hydrochloric acid each time. The hydrochloric acid extracts were freed of phenol with ether and the residual aqueous phase was adjusted to *p*H 5 with sodium hydroxide and concentrated at low temperature to about 25 cc. Two hundred and fifty cc. of absolute alcohol was added, and the precipitated impurities were filtered off and washed with alcohol. The filtrate was concentrated under reduced pressure to dryness and the residue was suspended in 150 cc. of 95% alcohol and refluxed for a few minutes. The suspension was cooled, filtered, and the filtrate was concentrated under reduced pressure to dryness. Preparations made in this fashion gave half-maximal growth of *L. casei* at a level of about 4 gamma per cc. In order for the alcohol extraction procedure to work satisfactorily it was necessary to use a relatively short period of digestion with trypsin and to forego the greater yield of activity attendant on more complete hydrolysis. Furthermore, before the concentrate was treated with phenol, the active material was quite insoluble in alcohol.

About half of the total activity of the digest remained in the non-dialyzable portion, and prolonged dialysis failed to remove it (compare refs. 4 and 6). However, if the non-dialyzable portion was adjusted to *p*H 4 and extracted three times with phenol, the majority of the active material remained in the aqueous phase, and was then readily dialyzable. By proceeding in this manner it was possible to prepare concentrates which gave half-maximal growth at 3–4 gamma per cc. of culture medium.

(b) By Separation with Heavy Metal Salts.—One hundred grams of vitamin-free casein were dissolved in 1 liter of water and enough sodium hydroxide to give a final *p*H of 8, 1 g. of trypsin (Fairchild) was added, and the mixture was held at 37° for twenty hours, 110 cc. of 20% lead acetate solution was added, the *p*H was adjusted to 8.5–9 by addition of sodium hydroxide, and the precipitate which formed was filtered off and washed. This and subsequent operations were conducted as rapidly as possible in order to minimize destruction of the strepogenin at elevated *p*H. The filtrate was treated with 400 cc. of a 20% solution of silver nitrate, the *p*H was again brought back to 8.5–9 with sodium hydroxide, and the precipitate which had formed was removed by filtration, and washed. Excess lead and silver ions were removed from the filtrate as chlorides, and the resulting acid filtrate was treated with a solution of 60 g. of mercuric chloride. The precipitate which formed was removed, and the filtrate was adjusted to *p*H 8.5–9 by addition of sodium hydroxide. The active material was precipitated by this manipulation, and was recovered from the alkaline mercury precipitate by suspension of the latter in water and decomposition with hydrogen sulfide, and removal of the mercury sulfide. Concentrates prepared in this manner showed about the same potency as those obtained by the phenol extraction methods.

Some Additional Properties of Strepogenin.—When concentrates of strepogenin were heated to boiling with 1 *N* sodium hydroxide or 6 *N* mineral acid for several hours the activity was reduced to 5–10% of that in the original concentrate. However, even after digestion overnight with 6 *N* hydrochloric acid some slight potency remained. In light of this fact the existence of traces of strepogenin in casein hydrolysates prepared by the action of hydrochloric acid was understandable (see above). Acetylation with acetic anhydride and sodium hydroxide in cold aqueous solution reduced the activity of purified concentrates to about 15% of that present originally. Chloramine T did not affect the potency. This fact was of interest since this reagent reacts readily in the cold with α-amino acids, but not with most peptides. Similarly, ninhydrin, which also reacts with amino acids, had relatively little inactivating effect on strepogenin. With this reagent at 100° for an hour the potency of a concentrate was reduced to about half.

Discussion

In the case of strepogenin we have an instance of a protein constituent which is probably not an amino acid (see below), and which is nevertheless of importance nutritionally with bacteria. Moreover, the strepogenin content of a protein is not reflected in the amino acid composition of that protein. For example, egg white which is well supplied with the known amino acids, is practically without strepogenin activity when digested with trypsin. Some proteins such as insulin are seen to be high in strepogenin. Furthermore, trypsinogen is considerably richer in this factor than is the very closely related trypsin.

The nature of the strepogenin molecule cannot be established before it is isolated in pure condition. However, certain aspects of its structure are apparent now. Evidence has been presented[4] which would indicate that strepogenin is an amphoteric substance. The failure of chloramine T to inactivate the factor, taken in connection with its lability to acid or alkali, and its occurrence in proteins, make it seem probable that it may be a peptide, rather than an α-amino acid. Its solubility behavior and its reaction toward various chemical reagents are likewise in line with the working hypothesis of the peptide nature for the compound.

Whether strepogenin is or is not a peptide, it nevertheless has a bearing on the structure of proteins. The relatively high content of the factor in very highly purified proteins would seem to argue against its presence as a chance contaminant. Likewise, the fact that pure proteins isolated from pancreas were far richer sources than was pancreas itself[7] points to the conclusion that strepogenin is a part of the protein molecule. Furthermore, slow liberation of the factor during tryptic digestion suggests that this compound is an integral part of the protein rather than an impurity. It would be possible for a protein to contain all the amino acids and yet not possess strepogenin activity, for the amino acids might not be linked together in the proper combination. This is one explanation for the absence of strepogenin from egg white and for the rather widely differing strepogenin contents of proteins of similar amino acid composition.

(7) In this connection, it was of interest to assay autolysates of pancreas, for it was found that a major portion of the strepogenin of this organ could be accounted for in the insulin and proteolytic enzymes normally found therein.

Summary

Tryptic digests of many highly purified proteins were found to be rich sources of the bacterial growth factor strepogenin. Crystalline insulin, crystalline trypsinogen, crystalline trypsin, crystalline chymotrypsins and chymotrypsinogen, crystalline ribonuclease, crystalline tobacco mosaic virus, certain crystalline proteins of yeast, hemoglobin, and casein were excellent or good sources. Dialyzed egg white, salmine, and gelatin were very poor sources. Comparative values for the strepogenin contents of tryptic digests of these proteins were determined. The rate of liberation of strepogenin activity by trypsin from different proteins varied, but the release was maximal for all in twenty hours or less under the conditions studied. Two independent procedures for the preparation of rather active concentrates of the growth factor from casein digest were described. Implications of the findings for the study of protein structure, and for the nutritional value of proteins to bacteria, were indicated briefly.

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[CONTRIBUTION FROM THE BAKER LABORATORY OF CHEMISTRY AT CORNELL UNIVERSITY]

Gliotoxin. VI. The Nature of the Sulfur Linkages. Conversion to Desthiogliotoxin[1,2]

BY JAMES D. DUTCHER,[3] JOHN R. JOHNSON AND WILLIAM F. BRUCE[4]

The early chemical investigations of gliotoxin ($C_{13}H_{14}N_2O_4S_2$) revealed that this substance is extremely sensitive to alkalies and is altered rapidly even by mild alkaline reagents such as sodium bicarbonate, sodium sulfite and sodium sulfide. These reagents evidently acted upon labile sulfur linkages in gliotoxin but elucidation of the chemical transformations involved has been hampered by the difficulty of isolating well-defined crystalline intermediate degradation products. One of the structural units of the gliotoxin molecule is probably an amino acid related to cysteine, and the complex character of the action of alkalies on cysteine and cystine is well known.[5]

The optical rotation and the antibiotic activity of gliotoxin are changed profoundly under the influence of alkaline reagents. Weindling[6] observed that the inhibitory action against rhizoctonia was lost rapidly even on standing in a buffered solution at *p*H 7.5, and we have found that the action of sodium bicarbonate, sodium bisulfite, and pyridine, also destroys the typical antibacterial activity.

In a previous paper[7] it was shown that exhaustive hydrolysis of gliotoxin with hot aqueous alkalies yields one of the nitrogen atoms as methylamine and the other as indole-2-carboxylic acid;

(1) A preliminary paper was presented at the Cleveland Meeting of the American Chemical Society, April 1944; see Abstracts of Papers, 107th Meeting, pp. 23-24M.

(2) Fifth paper, THIS JOURNAL, **67**, 423 (1945).

(3) Du Pont post doctorate Fellow; present address, Squibb Institute for Medical Research, New Brunswick, N. J.

(4) Present address: Wyeth Institute of Applied Biochemistry, 900 N. Broad Street, Philadelphia 30, Pa.

(5) Clarke and Inouye, *J. Biol. Chem.*, **89**, 399 (1930); **94**, 541 (1932); Zahnd and Clarke, *ibid.*, **102**, 171 (1933); Fruton and Clarke, *ibid.*, **106**, 667 (1934); see also Gortner and Sinclair, *ibid.*, **83**, 681 (1929).

(6) Weindling, *Phytopathology*, **24**, 1153 (1934).

(7) Bruce, Dutcher, Johnson and Miller, THIS JOURNAL, **66**, 614 (1944).