A STUDY OF BACTERIOLOGICAL MEDIA: THE EXAMINATION OF PEPTIDES IN BACTO-CASITONE*

BY A. F. S. A. HABEEB†

From the Sterling Chemistry Laboratory, Yale University, New Haven, Conn., U.S.A.

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Three batches of Bacto-Casitone have been fractionated by the combined use of paper electrophoresis at pH 5·8 and paper chromatography in butanol-acetic acid-water system. Batches A, B and C showed 61, 73 and 73 subfractions respectively. Some of the subfractions have been found to be a mixture of few peptides while others consist of a single peptide by *N*-terminal amino acid analysis.

IN a previous paper[1] it was found that the dinitrophenyl (DNP) peptides in Bacto-Casitone aggregated in four spots which were difficult to fractionate. This paper describes the fractionation of the peptides in three batches of casitone by the combined use of paper electrophoresis and paper chromatography. The homogeneity of each subfraction was tested by determining the *N*-terminal amino acid as DNP-derivative.

EXPERIMENTAL

The apparatus for paper electrophoresis was similar to that used by Kunkell and Tiselius[2] with some modifications. The buffer vessels 19½ in. × 3 in. × 4½ in. were made of lucite, the electrodes were platinum foil 2 in. × 1 in. The electrode vessels were connected to the buffer vessels by buffer bridges so as to prevent alterations in the electrolyte composition at the electrodes from reaching the filter paper. The paper was placed between two glass plates 19⅜ in. × 18 in. of 1·1 cm. thickness which were covered lightly with Dow Corning silicone grease. To get rid of heat produced during electrophoresis the lower glass plate rested on a metal box 15½ in. × 19 in. × 1 in. which was cooled by allowing water to flow through a coil in it. A sheet of Whatman 3 MM paper 9 in. × 22¼ in. was used, a line was drawn 10¼ in. from the anode side, then 0·3 ml. of 20 per cent solution of casitone batch A was applied as a streak on this line, the paper was sprayed with buffer pH 5·8 (pyridine: acetic acid: water, 8:2:90 v/v/v) leaving about 1½ in. from either side of the sample unsprayed. Then the buffer was allowed to flow from both sides so that the sample is concentrated in a thin band. Excess of buffer was blotted and the paper was placed between the glass plates for electrophoresis. The paper was left for half an hour for equilibration then 1000 V. was applied (current 50–70 mA.) for 4 hours. The paper

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† Present address: Division of Applied Biology, National Research Council, Ottawa, Ontario, Canada.

was then dried, a horizontal strip was cut from each end of the paper and the presence of the different fractions was revealed by the ninhydrin colour reaction. Using the two strips as guides vertical strips were cut from the remnant of the paper and each fraction was eluted with water. Two sheets were treated for each batch and eluates from similar fractions were pooled together. Each eluate was evaporated to dryness at room temperature in a vacuum desiccator. The residue was dissolved in 0·15 ml. of 10 per cent *iso*propanol.

Batch A gave 13 fractions, fractions F1–F6 are acidic in character, their mobilities decreases in that order, F7 is neutral, F8–F12 are basic with mobilities increasing in that order. Batches B and C gave similar results. Each fraction was subjected to electrophoresis at pH 5·8 to separate it from traces of neighbouring fractions. It was found that all fractions in addition to the main spot gave a second fraction in the neutral position designated Fx.

Each fraction was applied on Whatman No. 1 paper as 0·003 ml. spots 2 cm. apart. The papers were developed with the butanol-acetic acid-water system, then after drying two strips were cut from either end of the

TABLE I

ANALYSIS OF CASITONE

	Number of subfractions											
	Acidic fraction				Neutral		Basic fraction					
Batch	F3	F4	F5	F6	F7	Fx	F8	F8[1]	F9	F10	F11	Total
A	7	6	6	7	9	4	5	1	5	6	5	61
B	6	8	7	10	10	2	3	–	7	9	11	73
C	5	7	8	10	13	2	3	–	9	8	8	73

paper and the presence of the subfractions revealed by the ninhydrin colour reaction. Using the 2 strips as guides horizontal strips each representing a subfraction were cut and eluted with water. The eluate was evaporated to dryness in vacuum then dissolved in 0·1 ml. 10 per cent *iso*propanol. 0·05 ml. was completely hydrolysed with hydrochloric acid for 24 hours and after removal of hydrochloric acid, the residue was taken up in few drops of 10 per cent *iso*propanol and an aliquot was subjected to two dimensional chromatography for amino acids[3].

Determination of N-*terminal amino acid.* To 0·05 ml. of the subfraction was added 0·05 ml. of 2 per cent trimethylamine solution and 0·2 ml. of 5 per cent fluorodinitrobenzene (FDNB) in ethanol and the solution was shaken at room temperature for 2 hours. A few drops of trimethylamine solution and a few drops of water were then added and the excess of FDNB was extracted with ether. The aqueous layer was evaporated to dryness in a vacuum desiccator. The residue was taken up in a few drops of 6 N hydrochloric acid and hydrolysed for 8 hours at 105°. After removal of the hydrochloric acid it was subjected to two dimensional chromatography using the ethyl benzene system[4] followed by 1·5 M phosphate buffer.

TABLE II

THE HYDROLYSATES OF ACIDIC SUBFRACTIONS*

Subfraction	R_F	DNP-amino acid	Asp	Glu	Orn	Lys	Arg	His	Gly	Ala	Val	Leu	Ser	Thr	Tyr	Phe	Pro	Met
F3, 1	0·05–0·12	Leu, Ph, S, Val?	1	4	–	1	?	–	1–2	1–2	1–2	2	1–2	1	–	–	?	–
F3, 2	0·08–0·15	Ph	1	4	–	1	?	–	1–2	1–2	1–2	2	1	trace	–	–	?	–
F3, 3	0·08–0·16	Ph, S, Asp, Glu, Val	1	4	–	1	–	–	1–2	1	1	2	1–2	trace	–	–	–	–
F3, 4	0·16	Asp, Glu, Ph, Val, Thr, S, Ala?	1–2	4	–	trace	1	–	1	1	1–2	2	1	trace	–	–	1	1
F3, 5	0·21–0·39	Ph, Val, Asp, Glu, A, S, Ala?	1	4	–	–	–	–	1	2	2	2	1	1	–	–	1	–
F3, 6	0·31–0·53	Ph, Val, S	1	4	–	–	–	–	1	1–2	1–2	1–2	1	1	–	–	–	–
F3, 7	0·47–0·6	Ph	1	3	–	–	–	–	1	3	2	3	–	–	–	–	1	–
F4, 1	0·044–0·11	Ph, S	1	4	–	1–2	?	–	1	1	1	2	1	1	–	–	1	–
F4, 2	—	Ph, S, A	1	3	–	1	–	–	1–2	1	1	1–2	1	trace	–	–	1	–
F4, 3	0·17	Ph, S, Asp, Glu	1	3	–	1	–	–	1	1	1	2	1	trace	–	–	1	–
F4, 4	0·19–0·33	Ph, S, A, Val	1	2–3	–	trace	–	–	1	1	2	2	1	1	–	–	?	–
F4, 5	0·48–0·62	Ph, S, A, Val	1	3	–	–	–	–	1	1	1–2	1–2	1	–	–	–	1	–
F4, 6	0·48–0·62	Ph, S, A, Val	1	2–3	–	trace	–	–	1	1	1	1–2	1	–	–	–	1	–
F5, 1	0·1–0·14	Asp, Glu, S, Thr, Arg, Phe, Val, Lys, Gly?	1	4	–	1	–	–	1	1–2	1–2	2	1	1	–	–	1	–
F5, 2	0·10–0·14	Ph, S, Thr, Val, Arg	1	4	–	2	1	–	2	1–2	1	2	2	1	–	–	1	–
F5, 3	0·17–0·28	Ph, Val, Asp, Gly, S, Thr, Ala, Arg	1	3	–	1	1	–	2	2	2	3	2	1	?	–	1	–
F5, 4	0·27–0·45	Glu, Ph, Val	1	2	–	1	–	–	1–2	2	2	3	1–2	1	–	1	1	–
F5, 5	0·37–0·64	Val, Ph, A	1	2	–	trace	?	–	1–2	2	3	3	1	trace	–	–	2	1
F5, 6	0·48–0·68	Val, Ph, S, Arg	1	2	–	trace	1–2	–	2	2	3	3	–	–	–	1	1	1
F6, 1	0·06–0·13	Ph, S	1	3	–	2	1	?	1	1	1	2	–	trace	–	–	1	–
F6, 2	0·09–0·23	Ph, S, Thr?	1	2	–	1–2	1	–	1	1–2	1	1–2	trace	–	–	–	1	–
F6, 3	0·09–0·23	Ph	trace	2	trace	1	1	–	1	1	1	2	–	–	–	–	1	–
F6, 4	0·24–0·54	Ph	trace	2	trace	1	1	–	1	2	1	1	–	–	–	–	1	–
F6, 5	—	Ph	1	2	–	1	1	–	1	2	1	2	1	–	–	–	1	–
F6, 6	0·4–0·56	Ph	1	2–3	–	1	1	–	1	1	1	2	1	trace	–	–	1	–
F6, 7	0·4–0·56	—	1	3	–	1	?	–	1	1	2–3	2–3	1	–	–	–	1	–

* In the subfractions shown the first figure indicates the number of the fraction and the second is the number of the subfraction.
Ph represents spot below and slightly to the right of Phe.
A unidentified spot slightly below DNP-alanine position.
S represents serine or ε lysine.
? doubtful.

TABLE III

THE HYROLYSATES ON NEUTRAL SUBFRACTIONS*

Subfraction	*RF*	DNP-amino acids	Asp	Glu	Orn	Lys	Arg	His	Gly	Ala	Val	Leu	Ser	Thr	Tyr	Phe	Pro	Met
F7, 1	0·05–0·12	Asp, Glu, S, Ph, Lys?	1	3	–	2–3	1	–	1	2	1	2	1	1	–	–	1	–
F7, 2	0·08–0·12	Ph, S	2	2	–	1	–	–	1	1	1	1	1	–	–	–	1	–
F7, 3	0·18–0·27	Asp, Glu, S, Gly, Ala, Val, Ph, Thr? Lys	1	2	trace	1–2	1	?	2	2	1	2	2–3	1	–	–	1	–
F7, 4	0·20	S, Gly, Thr, Ala, Ph	1	2	–	1–2	1	–	1	1–2	1	2	1	1	–	–	1	–
F7, 5	0·25–0·38	Ph, S, Leu, Val, Ala, Thr, Lys	trace	1	–	1	1	–	1	4	1	2	1	1	–	–	1	–
F7, 6	0·32–0·38	Ph, S, Glu	1	2	trace	1–2	1	–	1	1–2	1	1–2	1	trace	1	1	1	–
F7, 7	0·4	Ph, Val, Met, Ala, S	1	2	trace	1	1	–	1	2–3	4	2	1	trace	1	–	1–2	2
F7, 8	0·43–0·59	Ph, Leu, Val, Ala, S, Thr, Pro	1	1	–	1	1	–	1	1	2	5	–	–	–	2	1	2–3
F7, 9	0·47–0·56	Ph, Leu, Val, Arg	1	2	–	1	1	–	1	2	2	5	1	1	–	2	2	2
Fx1	0·175	Ph, S	trace	1	–	–	1	–	1	1	–	–	1	–	–	–	–	–
Fx2	0·24	Ph, S	trace	1	–	–	1	–	1	1–2	–	–	1	–	–	–	–	–
Fx3	0·175	Ph, S	trace	1	–	–	1	–	1	1	–	–	1	–	–	–	–	–
Fx4	0·24	Ph, S	trace	1	–	–	1	–	1	1–2	–	–	1	–	–	–	–	–

* See footnote on Table II.

TABLE IV

THE HYDROLYSATE OF BASIC SUBFRACTIONS*

Subfraction	*RF*	DNP-amino acid	Asp	Glu	Orn	Lys	Arg	His	Gly	Ala	Val	Leu	Ser	Thr	Tyr	Phe	Pro	Met
F8, 1	0·14–0·26	Ph, S, Val	–	2	–	2	–	–	1	2	–	2	–	–	–	–	–	–
F8, 2	0·15–0·3	Ph	–	2	–	2	?	–	1	2	1	1–2	1	–	–	–	–	–
F8, 3	0·15–0·35	Ph, S	–	2	trace	2	–	–	1	1–2	1	1–2	1	–	–	–	–	–
F8, 4	0·18–0·35	Ph	–	1	–	1	1	–	1	1	1	1	1	–	–	–	–	–
F8, 5	0·43–0·51	Ph	–	1	–	1	1	–	1	1	1	1	1	–	–	–	?	–
F8[1]	0·38	Ph, Gly	–	1	–	1	1	–	1	1	2	1	1	–	–	–	1	–
F9, 1	0–0·04	Ph	1	1–2	–	2–3	–	–	1	1–2	1	1	1	1	–	–	–	–
F9, 2	0·07–0·12	Ph, S	trace	2	1	2–3	–	–	2	2–3	1	1	1	–	–	–	1	–
F9, 3	0·17–0·25	Ph, S, Lys	–	2	–	2	1	–	1	2	2	1	1	–	1	–	1	–
F9, 4	0·17–0·29	Ph, S, Ala	–	1	–	1	–	–	1	3	1	2	1	–	–	–	–	–
F9, 5	—	Ph	–	–	–	1	1	–	1	2	1	2–3	–	–	–	–	–	–
F10, 1	0–0·04	Lys, S, Ph	–	2	–	2–3	–	–	1	1–2	1	1	1	–	–	–	1	–
F10, 2	0·06–0·11	Lys, Ph, S	–	2–3	–	4	1	–	1	1–2	1	3–4	1	–	–	–	1	–
F10, 3	0·09–0·13	Lys, Ph, S, Arg	–	1–2	–	3	1	–	1	3	1	1	1	–	–	–	1	–
F10, 4	0·17–0·23	Ph, S, Glu?	–	1	–	2	–	–	2	2	1	2	1	–	–	–	1	–
F10, 5	0·17–0·29	Ph, S, Glu?	–	1	–	3	1	–	1	3	1	1	1	–	–	–	1	–
F10, 6	0·27–0·39	Ph, S, Lys	–	1	–	3	1	–	1	2	2	2	1	–	–	–	1	–
F11, 1	0·07–0·14	Phy, S, Lys, Arg	1	2–3	1	2–3	2	–	1	1–2	1	1	1	–	–	–	–	–
F11, 2	0·09–0·15	Ph, S, Lys, Arg, Thr?	–	1–2	–	2–3	1	–	1	1	trace	trace	1	1	–	–	–	–
F11, 3	0·19–0·24	Ph, Arg?	1	1	1	1	1	–	1	1–2	1	1	–	–	–	–	–	–
F11, 4	0–2–0·33	Ph, S, Val	–	1	–	2	1	–	1	1	1	2	–	–	–	–	–	–
F11, 5	0·31–0·35	Ph, Gly?	–	–	–	3	–	–	1	1	1	3	1	–	–	–	1	–

* See footnote to Table II.

Results

Fractions F1 and F2 contain free aspartic and glutamic acids respectively. F12 and F13 showed free lysine, arginine, histidine and arginine, lysine, ornithine, respectively.

Table I gives the numbers of subfractions obtained from each fraction of the three batches of casitone. F8[1] was a slow moving spot obtained when fraction F9 was subjected to electrophoresis.

Tables II–IV show the amino acid contents in the subfractions obtained from the acidic, neutral and basic fractions respectively for batch A Arbitrary figures ranging from 1–10 indicate the relative amounts of the amino acid judged from the size and intensity of the colour. The terminal amino acid of the peptide or peptides in each subfraction is also given as the DNP-derivative.

Batches B and C were similarly analysed for the *N*-terminal and the constituent amino acids of peptides in the subfractions. Some of the subfractions showed more than one *N*-terminal amino acid while others proved to be formed of only one peptide. An unidentified spot (Ph) appeared on the chromatogram of DNP-amino acids in the *N*-terminal position which occupied a position slightly below and to the right of phenylalanine. It may be an artifact.

Discussion

The electrophoretic patterns obtained with the three batches of casitone were similar, and demonstrated the fractionation of casitone into 11 fractions which were subjected to further separation by paper chromatography. Batches A, B and C gave 61, 73 and 73 subfractions respectively as seen in Table I.

The acidic subfractions showed a preponderance of aspartic and glutamic acid in their hydrolysates. The peptides examined had aspartic, glutamic acids, lysine, arginine and some of the neutral amino acids in the *N*-terminal position. The peptides in the basic subfractions were rich in lysine and arginine and some of them showed lysine, arginine and neutral amino acids as the *N*-terminal residue. Phosphopeptides have been separated from an enzymic hydrolysate of casein by Agren and Glomset[5]. Here phosphoserine peptides will show serine in the *N*-terminal position because DNP-phosphoserine gives DNP-serine on hydrolysis with 6N hydrochloric acid. A spot in the position of Ph has been reported previously as an artifact[6]. Some of the subfractions separated from casitone proved to be a mixture of peptides of similar mobility and R_F value as appears from the *N*-terminal amino acid residue, while others are formed of one peptide.

This analysis shows the complexity of the peptide mixture of casitone as noticed from Tables II–IV.

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