Chemical Nature of Toxohormone (Nakahara)

Eighth Report. Toxohormone AP II₁ and II' from Cancerous and Noncancerous Ascitic Fluids*

By

Hajime Masamune, Sigetosi Kamiyama, Sin-iti Abe,

(正宗 一) (加美山 茂利) (阿部 辰一)

Shoichi Abe, Mituo Haga and Hiroshi Kaketa

(阿部 正一) (芳賀 光男) (掛田 博)

From the Medico-chemical Institute, Tohoku University, Sendai

(Received for publication, January 15, 1959)

Formerly we¹⁾ mentioned that two toxohormones, one KIK-active and the other KIK-inactive, are secreted in urine of not only cancer patients but also normals. And, from gastric cancer as well as non-cancered gastric mucosa were isolated two different toxohormones²⁾, although both are group active mucopolysaccharides. Thus it might be said that generally one secretion or one tissue contains more than one kind of toxohormone. Regarding the cancerous ascitic fluid, we have detected so far three toxohormones in it, one of which is AP I³⁾ a globulin possessing simultaneously KIK potency, a second the KIK-active glucidamin reported by Sugimoto⁴⁾ and a third a globulin closely resembling AP I but KIK-inactive.

The present account deals with the last toxohormone from cancerous ascitic fluids with original seat in stomach (AP II₁) as well as a corresponding protein from ascitic fluids due to liver-cirrhosis as a representative kind of noncancerous ascitic fluid (AP II').

EXPERIMENTAL

Preparation Procedure of the Toxohormones

For each of the two kinds of toxohormone, one example of preparation runs will be described.

AP II₁, a toxohormone-active globulin other than AP I from cancerous ascitic fluids with original seat in stomach

First stage. 4.3 l. of combined cancerous ascitic fluids (light yellow, transparent; pH 8.2; d 1.015) from a stomach cancer patient was distilled in vacuo to about 800 cc., and the condensate was dialyzed against running water for 3 days, transferred to the middle compartment of a Pauli apparatus without antecedently separating off some precipitate, that had occurred

* 14th report of Masamune and co-workers' "On Proteins and Amino Acids".

during dialysis, and electrodialed until no more flow of electricity. The precipitate increased enormously thereby. The whole deposit was centrifuged, washed with ethanol and ether in succession and dried in vacuo over anhydrous calcium chloride. 42 g. of a gray powder (ascitic globulins =AG) was obtained.

Second stage. 42 g. of AG was pulverized, suspended in 600 cc. of distilled water, and after addition of 10% NaOH to pH 9.0, stood in a refrigerator (0–3°C) additionally adding the NaOH solution from time to time to maintain the pH value at 8.5–9.0. After 5 days, the mixture was supplemented with water to 800 cc. and centrifuged in a refrigerated centrifuge (0°C), and the opalescent supernatant (165 cc.) was neutralized with 1 *N* HCl and freed from some precipitate that had occurred thereby. 29.7 g. (18 g. per 100 cc. of the solution) of sodium chloride was added to the centrifuged supernatant (163 cc.), and this mixture was placed in the refrigerator for one day with occasional agitation and acidified to pH 4.2 with 1 *N* HCl. The abundant white precipitate was centrifuged off at 0°C and washed with two 60 cc. portions of 18% NaCl of pH 4.2. It was next suspended in 200 cc. of distilled water, adjusted to pH 8.5 with 5% NaOH and freed from sodium chloride by 3 days' dialysis against running water. The solution which had changed intensively cloudy was again alkalinized to pH 9.0, and the insoluble part was discarded after centrifugation. The supernatant here was neutralized with 2 *N* HCl exactly to pH 7.0, the small precipitate was eliminated, and after making up to 165 cc., the solution was subjected to alcoholic fractionation (next stage).

Third stage. 55 cc. of the neutral solution above was diluted to 100 cc. and chilled on ice, and abs. ethanol was added under vigorous agitation in such portions as each increased the ethanol concentration by 10% (v/v), centrifuging off every deposit in the refrigerated centrifuge after 2–3 hours. The centrifugates were separately washed with ethanol and ether and dried as above, with the yields of fractions:

Fraction No.	I	II	III	IV	V	VI	VII
Ethanol concn. (% (v/v))	0–10	10–20	20–30	30–40	40–50	50–60	60–70
Yield (mg.)	30	13	10	45	135	680	120

The balance of the solution at the end of the second stage was also treated in a similar manner, and the fractions which precipitated at 40–50% ethanol and those at 50–60% ethanol were united together. The total amount given was 2.4 g.

Fourth stage. As AG was in the second stage, 2.4 g. of the product was taken up in 200 cc. of water by means of alkali, and the part that was insoluble at pH 9.0 and that precipitated on shifting the hydrogen ion

concentration to pH 7.0 were eliminated. The light opalescent centrifuged supernatant (neutral) was made up to 240 cc. with water and fractionated by addition of 6.6 g. portions of solid ammonium sulfate, each of which increased the concentration of this salt by 0.2 *M*, in the manner alcoholic fractionation was carried out above. The fractions precipitated here were separately dissolved in 70–150 cc. of water and dialyzed against running water for 3–4 days to remove ammonium sulfate, employing toluene as a preservative. Then, the solutions were distilled under reduced pressure to syrupy consistency, dehydrated with excessive ethanol, washed with ethanol and ether and dried as above with yields:

Fraction No.	I, II, III	IV	V	VI	VII	VIII	IX
Molarity of ammonium sulfate (<i>M</i>)	0–0.4, 0.4–0.6, 0.6–0.8	0.8–1.0	1.0–1.2	1.2–1.4	1.4–1.6	1.6–1.8	1.8–2.0 2.0...3.0
Yield (mg.)	0	64	1080	373	297	85	75 157

Frs. V–VII were dissolved together in 130 cc. of water in the same manner as AG was in the second stage (Here almost no precipitate occurred when the solution of pH 9.0 was neutralized), and the neutral solution was made up to 160 cc. and fractionated by successive addition of 4.22 g. portions of ammonium sulfate as above. The deposits were freed from the salt, condensed, precipitated, washed and dried also as above. The yields here amounted as follows:

Fraction No.	I	II	III	IV	V
Molarity of ammonium sulfate (<i>M</i>)	0.6–0.8	0.8–1.0	1.0–1.2	1.2–1.4	1.4–1.6
Yield (mg.)	37	141	755	424	117

Fifth stage. 1.1 g. of united Fr. III and IV was suspended in 100 cc. of water, dissolved by the aid of NaOH also as AG was in the second stage and electrodialyzed, and the deposit thereby was centrifuged off, washed with water (about 60 cc.), and after again dissolving in 40 cc. of water by addition of 1 *N* NaOH to pH 7.4, freeze-dried. A white, light powder weighing 0.95 g. was given—**AP II₁**.

AP II', a corresponding protein fraction of ascitic fluids from liver cirrhosis patients as a representative kind of non-cancerous ascitic fluid

Starting with 1.6 *l.* of an ascitic fluid (yellowish, transparent; pH 8.2; *d* 1.015) from a liver-cirrhosis patient, 0.62 g. of a white, light powder corresponding to AP II₁—**AP II'**—was obtained in a manner similar to above without setting aside the intermediate products partially. The main part of the toxohormone here fell on the corresponding fractions to those above in fractionation with ethanol as well as in fractionations with ammonium sulfate.

Properties of the Preparatoins

1) *Homogeneity.* AP II₁ and II' gave each a single, symmetrical boundary after electrophoresis in either of an alkaline (pH 8.6) and an acid (pH 2.0) buffer. Their mobilities in the alkaline buffer were not the same. The examination was made in a Tiselius apparatus of HTD-1 type Hitachi. See Fig. 1.

Fig. 1. Electrophoresis patterns ($\times 2/3$) of 1% solutions in a veronal-HCl buffer of pH 8.6 and I 0.2 (A) and a glycine-HCl buffer of pH 2.0 and I 0.2 (B) of AP II₁ (a) and AP II' (b); current 5 mA; temperature 10°C; exposure 120 minutes after starting current. Mobility:

$$\begin{array}{ll}
 \text{AP II}_1 \text{ pH 8.6} & \frac{(-1.15) \times 0.01043 \times 0.18}{120 \times 60 \times 0.005} = -6.00 \times 10^{-5} \text{ cm}^2/\text{volt/sec} \\
 \text{pH 2.0} & \frac{(-0.80) \times 0.01544 \times 0.18}{120 \times 60 \times 0.005} = -6.18 \times 10^{-5} \text{ } \\
 \text{AP II}' \text{ pH 8.6} & \frac{(-1.08) \times 0.01043 \times 0.18}{120 \times 60 \times 0.005} = -5.63 \times 10^{-5} \text{ } \\
 \text{pH 2.0} & \frac{(-0.92) \times 0.01544 \times 0.18}{120 \times 60 \times 0.005} = -7.10 \times 10^{-5} \text{ }
 \end{array}$$

The homogeneity of the preparations was further ascertained by column chromatography:— A 1.1×20 cm. column of a strong anion exchanger Dowex 1 $\times$ 2 (200–400 mesh) was equilibrated with 0.025 M borax-HCl buffer of pH 8.5, and 2 or 1 cc. of the same buffer containing 20 mg. of AP II₁ or 10 mg. of AP II' respectively was added, and washed with also the same buffer at a flow rate of 40 drops per hour. The eluate was collected in fractions of 20 drops (about 2 cc.) and analyzed by a biuret reaction (Gornall *et al.*⁵⁾) and measurement of ultraviolet absorption at 280 m μ . The results are diagrammed in Fig. 2, showing a single symmetrical peak of concentration in a definite range of fractions for either of the substances. Namely, the preparations again proved homogeneous.

2) *Toxohormone activity.* It was assayed by our usual method³⁾. As shown in Table I, the minimum toxohormone active dosis of AP II₁ and II' were found about 2 and 4 mg. respectively.

Fig. 2. Column chromatograms of 20 mg. of AP II₁ (A) and 10 mg. of AP II' (B) developed by the aid of a Dowex 1×2 column. —○—○— Extinctions at 280 mμ. —●—●— Extinctions at 540 mμ after the biuret reaction.

TABLE I-A

Liver-catalase Depression *in vivo*

The average O₂ volume evolved for each dose in each test-set is expressed in per cent of the corresponding O₂ volume in the blank control.

Assay No.	Number of mice used	Amount of substance injected (mg.)			
		5	3	1	0.5
By AP II ₁ :					
1	12		62.8	76.0	91.4
2	9		58.8		70.9
3	9		65.3	80.3	
4	9			76.6	82.2
5	6		72.7		
6	6			79.1	
7	9			83.5	91.1
Average			64.9	79.1	83.9
By AP II' :					
8	9	74.4		75.2	
9	12	63.4	77.9	85.3	
10	12	64.8	70.3	86.1	
11	9	68.5	82.7		
12	9	71.4	73.2		
Average		68.5	76.0	82.2	

TABLE I-B
Effect *in vivo* on Kidney- and Blood-catalase

Assay No.	Kidney	Blood	Liver
Effect of AP II₁ :			
<i>Oxygen evolved (cc.) in blank experiment</i>			
201	5.2	1.6	15.8
202	5.3	1.9	15.5
203	3.5	2.2	12.3
Average	4.3	1.9	14.5
<i>Oxygen evolved (cc.) in main experiment</i>			
204	4.0	1.9	10.9
205	4.9	1.7	10.6
206	5.1	2.0	10.2
Average (% of control value)	4.7 (109)	1.9 (100)	10.6 (73)
Effect of AP II' :			
<i>Oxygen evolved (cc.) in blank experiment</i>			
207	5.1	2.4	13.5
208	5.8	2.0	13.3
209	4.4	2.4	14.6
Average	5.1	2.3	13.8
<i>Oxygen evolved (cc.) in main experiment</i>			
210	4.5	2.3	11.2
211	5.3	2.2	7.9
212	5.7	2.5	10.7
Average (% of control value)	5.2 (102)	2.3 (100)	9.9 (72)

3) *KIK activity.* The method Masamune and Kawasaki described⁶⁾ was applied. The result is illustrated in Table II. The substances are regarded as KIK-inactive, because neither of them induced anemia in rabbits even when injected intravenously in dosage of 1.5 mg. per kg. body weight.

4) *Paper chromatography of the sugars and amino acids constituting the preparations.* a) Sugars. 10.6 mg. of AP II₁ was heated with 1 cc. of 1 *N* sulfuric acid in a sealed tube at 100°C for 5 hours. About 3.5 cc. of the satd. baryta was added to the hydrolysate until a pH value of 4.0, and the

TABLE I-C
Effect *in vitro* on Liver-catalase

Assay No.	Liver-catalase activity (oxygen evolved in cc.)		
	Control I (No incubation)	Control II (Incubated without toxohormone)	Main (Incubated with toxohormone)
Effect of AP II₁ (3 mg.):			
301	13.8	11.8	12.0
302	12.5	9.0	8.9
303	16.5	10.5	10.3
Average	14.3	10.4	10.3 (99% of Control II value)
Effect of AP II' (5 mg.):			
304	16.5	11.3	10.8
305	15.2	11.5	11.8
306	12.0	8.4	9.2
Average	14.6	10.4	10.6 (102% of Control II value)

TABLE II
Anemia-inducing Potency of AP II₁ and II'

Assay No.	Rabbit			Amount of substance injected* per kg. body weight (mg.)	Number of red cells ($\times 10^4$, per mm ³)						
	No.	Body wt. (kg.)	Sex		Before inj.	Time after inj. (hrs.)					Maximum decrease (%)
						1	2	3	4	5	
The potency of AP II ₁ :											
1	35	2.0	♀	1.5	551	568	559	601	540	534	4
2	36	2.2	♂	1.5	533	521	544	479	496	492	8
The potency of AP II' :											
3	35	2.0	♀	1.5	590	588	579	589	587	569	4
4	36	2.2	♂	1.5	750	790	739	757	777	755	1

* Intravenously.

precipitate was centrifuged off and discarded after washing with warm water. The supernatant and washing were evaporated to about 1 cc. by standing in vacuo over anhydrous calcium chloride, and the deposit occurring thereby was filtered off and washed. The filtrate and washing here were distilled to dryness, and the still-residue was dissolved in 0.1 cc. of

Fig. 3. Sugar paper chromatograms ($\times 2/5$) of the hydrolysates of AP II₁ (A) and of AP II' (B) developed with solvent butanol-pyridine-water (5:3:2); slivers 60×7 cm., Toyo Roshi filter paper; irrigation descending; temperature of test: AP II₁ $25^\circ \pm 2^\circ\text{C}$, AP II' $22^\circ \pm 1^\circ\text{C}$; duration of test: AP II₁ 20 hrs., AP II' 24 hrs. Reference runs effected with 1% solutions with respect to individual sugars. Indicator aniline hydrogen phthalate reagent of Partridge.

a, b: reference. *s*: main run. 1 galactosamine, 2 glucosamine, 3 galactose, 4 mannose, 5 L-fucose, 6 glucose, 7 xylose.

water and paper-chromatographed according to Masamune and Yosiwaza⁷⁾. As regards AP II', 18.2 mg. of it was hydrolyzed, and the still-residue of the hydrolysate free from H_2SO_4 was dissolved in 0.1 cc. and chromatographed taking 0.04 cc. of the solution. See Fig. 3.

In either of the chromatograms, spots were shown at the positions corresponding to galactosamine, glucosamine, galactose and mannose. It is doubtful that the very faint spots at the positions corresponding to glucose are plausibly ascribed to this sugar.

b) Amino acids. 5.6 mg. of AP II₁ was sealed with 1 cc. of 6 *N* HCl in an ampoule and heated in a boiling water-bath for 24 hours. The hydrolysate was freed from the hydrochloric acid by repeated vacuum evaporations over solid caustic soda to dryness, adding water to every residue. The final evaporated residue was taken up in 0.18 cc. of water (The solution was not perfectly clear), and 0.03 cc. of the solution was chromatographed on a 30×30 cm. square of Toyo Roshi filter paper No. 50. Of AP II', 6 mg. was hydrolyzed, and the still-residue of the hydrolysate freed from the free hydrochloric acid was dissolved in 0.24 cc. of water and chromatographed taking 0.04 cc. of the solution. Aspartic and glutamic acids, serine, glycine, threonine, alanine, tyrosine, phenylalanine, leucine and/or isoleucine, valine and/or methionine, proline, hexone bases and a

Fig. 4. Two-dimensional amino acid-paper chromatograms of the hydrolysates of AP II₁ (A) and of AP II' (B), developed by employing solvent butanol-acetic acid-water (4:1:2) for the primary irrigation and solvent phenol-0.1 M borax (10:1) for the secondary irrigation. Paper square 30×30 cm., Toyo Roshi filter paper No. 50. Temperature of test 20–25°C. Spray reagent 0.2% ninhydrin in moist butanol.

1 aspartic acid, 2 glutamic acid, 3 serine, 4 glycine, 5 threonine, 6 alanine, 7 tyrosine, 8 valine and/or methionine, 9 phenylalanine, leucine and/or isoleucine, 10 proline, 11 hexone bases, 12 a probable peptide.

probable peptide were detected in AP II₁ as well as in AP II'.

5) *Test-tube tests.* Both of the preparations were strongly biuret positive and weakly Molisch positive.

6) *Quantitative analyses.* The analytical figures are embodied in Table III. No quantitative discrepancies are discerned between AP II₁ and II' regarding hexosamine and hexoses, because these components are too small in content to give reliably accurate values. With respect to the amino acids, serine is present less and the total sum of phenylalanine, leucine and isoleucine as expressed as leucine more in AP II₁ than in AP II'.

7) *Ultraviolet absorption.* 0.5 mg% solutions in water were examined in a photoelectric spectrophotometer of EPU-2A type Hitachi. As shown in Fig. 5, ultraviolet absorption with maximum at 278 mμ occurred in similar strengths by the two proteins.

8) *Specific refraction.* 1% solutions (pH 8.0) of the preparations were examined in an Abbe refractometer at 17.5°C, giving resembling $[n]_D^{17.5}$ values:—

TABLE III
Composition in per cent of AP II₁ and AP II'

Analysis	AP II ₁	AP II'
N*	14.4	14.4
Ash†	0.8	1.6
Hexose‡ (as galactose)		
{ PHR ₂	2.1	1.4
{ SMHR ₂	0.8	0.4
{ HR ₁₀	2.5	1.8
Hexosamine§	2.3	1.6
Pentose//	0	0
Methylpentose¶	0	0
Sialic acid**		
{ ER	0	0
{ DR	0	0
Amino acids††:		
Aspartic acid	14.8	16.2
Glutamic acid	14.8	15.4
Serine	11.6	14.3
Glycine	6.4	7.5
Threonine	5.8	6.4
Alanine	6.2	5.8
Tyrosine	3.2	2.9
Valine and methionine (as valine)	7.0	6.4
Phenylalanine, leucine and isoleucine (as leucine)	16.0	12.2
Proline	6.6	6.0
Hexone bases (as lysine)	7.5	6.8

* Micro-Kjeldahl method. † Pregl method without use of H₂SO₄.
‡ Masamune and Sakamoto⁹. § Masamune and Yosizawa⁹. // Masamune and Sakamoto⁹. ¶ Masamune and Kawasaki¹⁰. ** Werner and Odin (direct Ehrlich method and diphenylamine method)¹¹. †† A modification of Masamune and Hakomori method¹² (Solvent phenol-0.1 *M* borax (10:1) was replaced for solvent phenol-0.1 *M* borax (86:14)).

AP II₁:

$$n=1.33513, \quad n_0=1.33324, \quad d^{17.5}/_4=1.0035, \quad d_0^{17.5}/_4=1.0003$$

$$[n]_D^{17.5} = \frac{1.33513^2-1}{1.33513^2+2} \times \frac{1}{1.0035} \times \frac{100}{1.000} - \frac{1.33324^2-1}{1.33324^2+2} \times \frac{1}{1.0003} \times \frac{99}{1.000} = 0.245$$

AP II':

$$n=1.33547, \quad n_0=1.33324, \quad d^{17.5}/_4=1.0035, \quad d_0^{17.5}/_4=1.0003$$

$$[n]_D^{17.5} = \frac{1.33547^2-1}{1.33547^2+2} \times \frac{1}{1.0035} \times \frac{100}{1.000} - \frac{1.33324^2-1}{1.33324^2+2} \times \frac{1}{1.0003} \times \frac{99}{1.000} = 0.264$$

In short, the cancerous toxohormone here and the corresponding

Fig. 5. Ultraviolet absorption spectra of 0.5 mg% aqueous solutions (pH 6.6) of AP II₁ (—○—○—) and of AP II' (—●—●—).

noncancerous differ in composition in serine content, phenylalanine, leucine and isoleucine content as a whole and possibly also in electrophoretic mobility in so far as was examined chemical-analytically and physically.

SUMMARY

1. There have been isolated toxohormones AP II₁ and II' which are globulins in the cancerous ascitic fluid of stomach cancer patients and noncancerous ascitic fluid of liver cirrhosis patients respectively and both precipitate at 40 to 60% ethanol and at 1.0–1.4 *M* ammonium sulfate.
2. They were examined biologically, chemically and physically.
3. They are devoid of anemia-inducing potency.
4. The former protein is more strongly toxohormone active than the latter, while they differ from each other only regarding serine content and phenylalanine, leucine and isoleucine content as a whole and possibly also regarding electrophoretic mobility, in so far as was examined.

The Ministry of Education gave a grant in aid to us through the Grant Committee for Medical, Dental and Pharmaceutical Researches, which is gratefully acknowledged. H. Masamune.

References

- 1) Masamune, Kaketa & Abe, S., *Tohoku J. Exp. Med.*, 1958, **67**, 329 ; Masamune, Hakomori, Kaketa & Abe, S., *ibid.*, 1958, **67**, 334 ; Masamune, Hakomori, Kaketa, Sinohara & Abe, S., *ibid.*, 1958, **68**, 63.
 - 2) Masamune, Kawasaki, Abe, S., Oyama, & Yamaguchi, *ibid.*, 1958, **68**, 81 ; Kawasaki, *ibid.*, 1958, **68**, 119 ; 1959, **69**, 153.
 - 3) Masamune, Tsuiki, Kamiyama, Abe, S., Haga, Kawasaki, Kaketa & Abe, Sh., *ibid.*, 1958, **67**, 309.
 - 4) Sugimoto, *ibid.*, 1956, **64**, 271 ; Masamune, Hakomori, Sugimoto & Genda, unpublished.
 - 5) Gornall, Bardawill & David, *J. Biol. Chem.*, 1948, **177**, 751.
 - 6) Masamune & Kawasaki, *Tohoku J. Exp. Med.*, 1956, **63**, 369 (f. 372).
 - 7) Masamune & Yosizawa, *ibid.*, 1953, **59**, 1.
 - 8) Masamune & Sakamoto, *ibid.*, 1956, **63**, 345.
 - 9) Masamune & Yosizawa, *ibid.*, 1957, **65**, 169.
 - 10) Masamune & Kawasaki, *ibid.*, 1956, **63**, 369 (f. 381).
 - 11) Werner & Odin, *Acta soc. med. Upsal.*, 1952, **57**, 270.
 - 12) Masamune & Hakomori, *Tohoku J. Exp. Med.*, 1956, **64**, 59.
-