FLOCCULATION REACTION IN A SYSTEM HUMAN GROWTH HORMONE – ANTIHORMONAL HORSE SERUM

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A new flocculation reaction between human growth hormone and antihormonal horse serum may be used for quantitative assay of this hormone in pituitary extracts and finished preparations.

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Immunologic and immunochemical methods are now widely used to investigate and, more particularly, to determine protein hormones, especially growth hormone (STH): precipitation [2, 10], ring-precipitation [8], anaphylaxis [8, 11], complement fixation [6, 15], hemagglutination, inhibition of hemagglutination [3, 14, 17], and, finally, radioimmunological reactions [7, 16]. However, there is one further immunochemical method (based on flocculation of the antigen – antibody complex) whose application to hormone investigation has not yet been described. This is because so far only non-flocculating antisera of small animals have so far been used in hormone immunochemistry. We have obtained an antisomatotropic horse serum, capable of flocculation. It should be remember that for some unknown reason, although rabbit antisera give flocculation, by no means all horse antisera have this property. After Ramon's discovery of the flocculation reaction of diphtheria and tetanus antisera in 1922, only a few examples have been added to the list of flocculating systems [13]. In the present investigation, the possibility of using the flocculation reaction for STH assay was studied.

EXPERIMENTAL METHOD

Antisomatotropic (anti-STH) serum was obtained by immunizing horses with microdoses of human growth hormone prepared by Roben's method [12]. The total dose of hormone used for immunization of a horse was 30-35 mg. The titer of passive hemagglutination was 1 : 6400–1 : 12,800. The antiserum formed one precipitation line in agar gel and possessed high hormonal and species specificity. One sample of antiserum (0.12 ml) completely neutralized 40 μg hormone in the tibia test on hypophysectomized rats. The method of obtaining anti-STH horse serum and its properties have been described fully earlier [4].

The flocculation reaction was carried out in tubes containing equal amounts of STH, and successively increasing amounts of antiserum. To equalize the volume and pH conditions, phosphate buffer was added to the tubes. The contents of tubes were mixed in the cold and transferred to a water bath at a constant temperature of 45-60°.

EXPERIMENTAL RESULTS

The flocculation reaction between STH and anti-STH serum developed in the following order. At first an increase of opalescence was observed, after which pinpoint floccules formed in the layer next to the wall; at a certain moment these floccules rapidly enlarged and spread throughout the layer of liquid. This moment was counted as the flocculation time. Flocculation began first in the initial tube in which antigen and antibody were present in an equivalent, mutually neutralizing ratio [1, 5, 13]. A slight excess of hormone or antiserum inhibited flocculation, and if one component was present in considerable excess no flocculation took place. Knowing the hormone content in the initial tube, the titer of antiserum could thus be determined, and conversely, knowing the titer of antiserum previously established relative to a hormonal standard, the content of hormone in a test sample can be determined.

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TABLE 1. Flocculation Reaction in System STH – Anti-STH at 45°

	Tube No.					
	1	2	3	4	5	6
STH (in mg/ml)	0,15	0,15	0,15	0,15	0,15	0,15
Phosphate buffer, pH 7.5 (in ml)	0,35	0,30	0,25	0,20	0,15	0,10
Antiserum (in ml)	0,30	0,35	0,40	0,45	0,50	0,55
Flocculation time (in min)	35	30	27	25	26	28

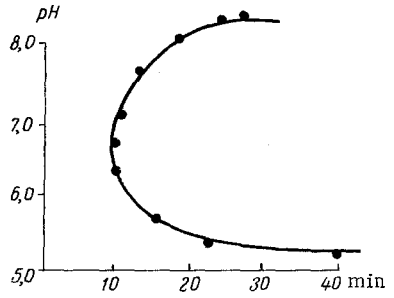

Fig. 1. Effect of pH of medium on rate of flocculation. STH content 0.15 mg, antiserum 0.4 ml. Volume of mixture together with phosphate buffer corresponding to pH value 0.8 ml. Temperature 45°.

It is clear from Table 1, that tube No. 4 was the initial one; in it 0.15 mg STH neutralized 0.45 ml antiserum quantitatively, in full agreement with the result of biological neutralization in the tibia test for this series of preparations [4].

The flocculation reaction is highly dependent on the conditions under which it is performed, the concentrations of the components, the temperature, and the pH and composition of the medium. The higher the concentration of STH, the shorter the flocculation time. With an STH concentration of 0.05 mg/ml, the reaction took 145 min to reach completion, compared wtih only 15 min for 0.25 mg/ml. The pH optimum for flocculation was 6.3-6.5 (Fig. 1). At pH values below 5.3 and above 8.3, the reaction velocity fell sharply, and with further acidification or alkalification no flocculation took place. Sodium, potassium, and ammonium chlorides inhibited the reaction, while sulfates inhibited it only in concentrations below 0.2 M, after which they accelerated it to an increasing degree as the concentration arose (Fig. 2). Acetone (15%) almost doubled the flocculation time, but in a higher concentration it stimulated the reaction on account of its side effect of precipitating protein. Ethanol acted in the same way in a concentration of 5%. A low temperature also inhibited the reaction. At 45°, the flocculation time was 25 min, rising to 115 min at 20° and to several hours or even days at 10° or lower. Conversely, at 60° flocculation was complete within 8 min, and at 65-70° within 3-4 min. At temperatures above 70°, the protein coagulated.

Flocculation is the most thermolabile of all known immunologic reactions. If an STH solution in a concentration of 1 mg/ml was heated at pH 7.5 on a boiling water bath for 1 min only, on the subsequent addition of an equivalent dose of antiserum no flocculation took place. Boiling the same solution of hormone for 30 min merely reduced the intensity of the precipitation and hemagglutination reactions (Table 2). It is interesting to note that the biological activity of STH persists even after boiling its solution for 10 min [3]. Consequently, flocculation can take place only with a biologically active hormone, a matter of fundamental importance when discussing the possibilities and limitations of the use of a particular immunologic method for hormone assay.

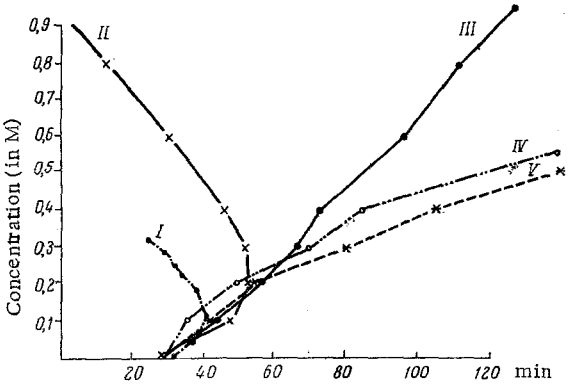

Fig. 2. Relationship between flocculation and mineral composition of medium. STH content 0.15 mg, antiserum 0.5 ml. Solution of mineral salt of appropriate concentration in phosphate buffer, pH 7.5. I) $ZnSO_4$, II) $(NH_4)_2SO_4$, III) NaCl, IV) KCl, V) NH_4Cl.

TABLE 2. Effect of Preliminary Heating of STH Solution (1 mg/ml, pH 7.5) on Intensity of Immunologic Reactions

	Intensity of reaction (in %)				
		after heating for			
	before heating	1 min	10 min	20 min	30 min
Flocculation	100	0	0	0	0
Precipitation	100	100	80	50	30
Hemagglutination	100	100	70	60	40

Note. Heated on boiling water bath.

Because of the special features of flocculation described above, it can be recommended for quantitative assay of growth hormone in pituitary extracts at the fractionation stage and in the finished product. The conditions under which this reaction can be used for STH analysis in biological fluids are being studied. Our recommendation is strengthened by the exceptionally high specificity of flocculation. With antigen concentrations of between 5 mg and 5 μg, horse antiserum against human growth hormone flocculates only with human and monkey STH, and does not flocculate with ACTH, thyrotropic hormone, chorionic hormone, and other human or monkey tissue or serum antigens, or with STH and other hormones of animal origin. Meanwhile, none of the hormonal antigens, including human or monkey STH, flocculate with rabbit anti-STH serum or with normal or antitoxic horse serum.

The resolving power of flocculation with visual observation is 10–5 μg hormone/ml. The error of the method is limited by the experimenter's ability to determine reliably the difference between two reactions in two neighboring tubes, and for the system given in Table 1, it is ±10%.

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