PREPARATION OF A HIGHLY PURIFIED HORSE ANTISERUM AGAINST HUMAN GROWTH HORMONE

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A method is described for the isolation of globulins free from contamination with albumins from horse antiserum against human growth hormone followed by their purification by enzymic hydrolysis, thermal denaturation, and fractionation on Sephadex G-75. The antibody titer per gram protein is increased 4 times and the antibody concentration is increased 6 times.

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A method of obtaining horse antiserum against human growth hormone possessing unique properties of flocculation was described previously [4]. By the use of this serum a new rapid method of quantitative analysis of growth hormone in pituitary extracts and finished products developed [5]. According to another report, partial purification of the antiserum by alcoholic fractionation considerably increases the specificity and reliability of determination of somatotropic hormone (STH) in biological media by the method of inhibition of hemagglutination [6]. However, the antisomatotropic γ-globulin produced in this way has a high molecular weight (about 160,000) and contains inactive impurities.

In this paper an improved method of purification of horse antisomatotropic serum by gentle hydrolysis with pepsin followed by fractionation of the hydrolysate with ammonium sulfate and gel-filtration is described.

In the case of antitoxic sera it is known that pepsin produces more intensive hydrolysis of inert proteins, accompanied by a decrease in the molecular weight of the antibodies, leading to a decrease in their species-specificity [2]. This last feature is particularly important for the use of antigrowth serum for the purpose of neutralizing the stimulant effect of growth hormone on the development of neoplasms [1, 3].

Native antisomatotropic serum was obtained by immunization of horses with microdoses of antigen [4]. Its activity and the yield of activity at the various stages of purification were determined by flocculation [5]. Those batches of sera which, in a volume of 0.6-0.4 ml, neutralized 0.15 mg of a laboratory standard STH were purified. The standard STH (batch No. 89), in a total dose of 40 μg, in the tibia test on hypophysectomized rats [9] increased the width of the cartilage to 255±1.9 μ compared with 158±2.9 μ in the control (physiological saline).

To compare the various conditions of isolation of the globulins and the methods of their subsequent purification, the same batches of native serum were used. Hydrolysis was carried out with pepsin together with a filler prepared at the Moscow Meat Combine specially for purification of antisera. Sephadex G-75 was used for gel filtration. Electrophoresis on paper was carried out on the UÉF apparatus under the conditions described in the instructions supplied with the apparatus (Kiev, 1964).

The antisomatotropic serum was purified as follows. Native serum of known titer (sample for analysis No. 1) in a volume of 1 liter was mixed with 240 g ammonium sulfate and kept in the cold for 3 h. The resulting precipitate of globulins was centrifuged or filtered through calico and washed with 0.5 liter of 24% ammonium sulfate solution. After washing, the precipitate was pressed and dissolved in distilled water up to a volume of 0.9 liter, after which sample No. 2 was taken from the solution.

The solution was acidified with 1N HCl to pH 3.5 and mixed with 20 ml 5% phenol and 10 g pepsin, the mixture being heated on a water bath to 30°. After mixing for 2 h sample No. 3 was taken, and immediately neutralized with a drop of alkali to stop the action of the enzyme.

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TABLE 1. Activity of Globulin Fractions of Antisomatotropic Serum Depending on Salting Out Conditions

	Quantity of $(NH_4)_2SO_4$ (in g/liter)					
	200	220	240	260	280	300
Relative percent of protein	61,2	70,3	84,8	89,7	95,7	100
Percent of activity by flocculation test	56,3	69,2	84,9	90,0	100	100

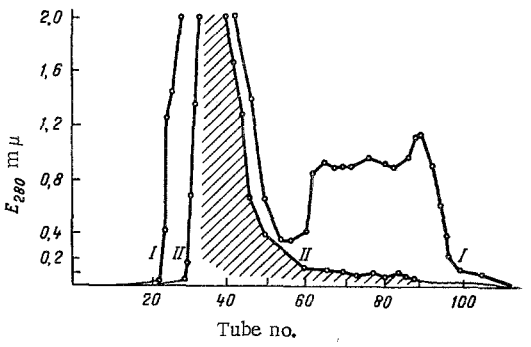

Fig. 1. Combined graph of two gel filtrations of hydrolyzed antisomatotropic globulins. Column (4.8 × 50 cm) with Sephadex G-75. I) Separative filtration of 20 ml 4% solution of active globulins in 35% ammonium sulfate. Active protein in tubes Nos. 22 and 51, each in a volume of 10 ml. Zone of ammonia Nos. 61-95; II) gel filtration of 10 ml 4% preliminarily dialyzed globulins (shaded). Activity in tubes Nos. 30-48, followed by low-molecular weight inactive fractions.

After enzymic hydrolysis the solution was mixed with 140 g/liter ammonium sulfate, the pH adjusted to 4.3-4.5, and heated for 15-20 min to 56°, mixing being continued for a further 45 min at this temperature. The mixture was then cooled to 18-20°, and then centrifuged or filtered. The residue of thermolabile proteins was washed with 0.3 liter 14% ammonium sulfate solution, pressed, and discarded. The main supernatant and the washings were pooled, their pH adjusted to 7.0-7.1 (sample No. 4), and mixed with 200 g/liter ammonium sulfate. The mixture was allowed to stand in the cold until the precipitate of active globulins was fully formed.

Thereafter the residue was treated by two methods: by ordinary dialysis as used in the formula for purification of antitoxic sera [2, 7], or by gel-filtration of a solution of globulins through Sephadex G-75. The latter method of removal of ammonium sulfate, combined with additional purification to remove inert low-molecular weight products of proteolysis has not previously been used with immune sera, so that it will be described fully below.

The residue of active globulins was dissolved in a minimal volume of distilled water with alkalification to pH 7.1-7.2, added to 0.05% chloroform, and transferred to a column with Sephadex G-75, equilibrated with neutral water. As soon as the solution had passed into the gel, an equal volume of water was added to the column, and after this had been absorbed, continuous elution with neutral water began. The yield of protein in the various fractions was verified spectrophotometrically, the activity was determined by flocculation, and ammonia by Nessler's method. Antisomatotropic globulin was removed from the column in the first concentrated fraction, considerably before the ammonia front (Fig. 1). The active fraction was collected and lyophilized; when necessary the powder was dissolved in physiological saline, sterilized, and poured into ampules, the solution being standardized as protein and by its ability to neutralize growth hormone in the flocculation test.

In contrast to the "Diaferm" method used to purify antitoxic sera, in which the material hydrolyzed by the enzyme consists of serum or "total globulins" [2, 7], which in fact, as the results of electrophoresis show (Fig. 2, No. 2), contain almost the same quantity of albumins as native serum (Fig. 2, No. 1), in this case we treated with enzyme globulins completely freed from contamination with albumins (Fig. 2, No. 4). To do this, 240 g ammonium sulfate was added to one liter native serum. As the quantity of ammonia decreased, the loss of activity increased sharply, while with an increase in ammonia up to 260 g/liter, albumins began to pass into the residue (Fig. 2, No. 3) Under optimal conditions of salting out of the globulins (240 g/liter), up to 15% of the activity was lost (Table 1), but this loss was only temporary. The fact is that under the conditions used in the "Diaferm" method, the pepsin hydrolyzes only 20% of the albumin, and most of it is converted under these circumstances into material with the electrophoretic mobility of globulins [10]. Because of this the peak of globulins is increased not only in the stage of fermentation, but also during subsequent thermal denaturation [8].

TABLE 2. Yield of Activity at Stages of Purification and Degree of Purification of Antisomatotropic Serum in Relation to Method Used

Method	Activity at stages of purification (in percent)					Coefficient of purification	
	separation of globulins	fermentation	thermal denaturation	dialysis	final yield	initial	in final product
"Diaferm"	–	78.7	77.5	71.0	43.3	1	3.1
"Total globulins"	100	75.7	80.3	73.8	44.7	1	3.2
Personal	85.7	82.6	81.0	85.5	49.0	· 1.5	3.6

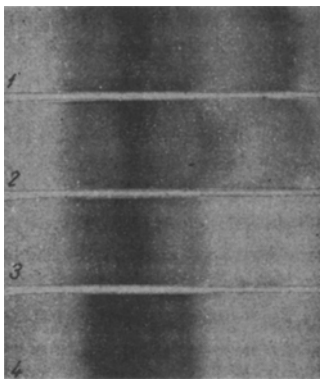

Fig. 2. Electrophoresis of native antiserum and of globulins isolated by ammonium sulfate. Description in text.

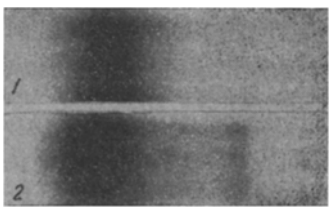

Fig. 3. Electrophoresis of purified antisera. Description in text.

The additional globulins (preformed albumins) are salted out along with the active fraction, and this complicates the purification. In my method of purification this does not happen, because the albumins do not take part in the fermentation, so that loss of activity at the stage of preliminary removal of albumins is compensated in the subsequent stages of purification of the active globulins (Table 2). The degree of purity of the finished product is also increased, up to 3.6 or more (compared with 3.1-3.2 for serum purified by the "Diaferm" method or by the "total globulins" method). This fact is also confirmed by electrophoretic analysis of the finished products (Fig. 3). Serum purified by the "Diaferm" method is definitely contaminated by the second fraction (Fig. 2, No. 2), while serum purified by my method has only one fraction (Fig. 2, No. 1).

In one variation of the method described, gel-filtration on Sephadex-G-75 was used. In this method, ammonium sulfate and low-molecular weight inactive products of proteolysis, which could not be completely removed by thermal denaturation, were removed simultaneously. Two gel filtrations are plotted on the same graph in Fig. 1: I demonstrates filtration of a solution of active globulins containing ammonium sulfate, II demonstrates filtration of globulins preliminarily treated by ordinary dialysis. Two main peaks are visible in the first graph, one of which contains all the active globulin, completely freed from ammonium sulfate, while the second consists entirely of ammonium sulfate. However, in the zone of ammonia, as the shaded graph clearly shows, very small residual peaks of low-molecular weight proteins not showing activity are also present. The finished product of purified antisomatotropic serum includes material of the first peak, collected between tubes Nos. 24 and 50. The yield of active material from the column was 85-90%. On refiltration through Sephadex G-75 under the same conditions, one symmetrical peak was obtained. In the "Diaferm" method, the processes of dialysis and additional purification are separate. The ammonium sulfate is first removed by ordinary dialysis against tap water, after which the additional purification of the active globulins is carried out by treatment with chloroform and isosedimentation.

For technical reasons large batches of antisomatotropic serum (50 liters per batch) were purified after preliminary isolation of the globulins with ammonium sulfate (240 g/liter), but followed by processing of the purified globulins to the finished state by filtration without gel. The mean protein content of the finished sterile products was 15%, its coefficient of purity 3.5-4.0, and its coefficient of concentration 5-6. On the average in the flocculation test 0.07-0.1 ml of the product fixed 0.15 mg of standard STH.

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