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APPLICATION OF IMMUNOADSORBENTS FOR ISOLATION OF PLACENTAL ALKALINE PHOSPHATASE, CARBOXYPEPTIDASE G-1, AND SERUM HEPATITIS ANTIGEN

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INTRODUCTION

The extraordinary specificity of antigen-antibody reactions forms the basis of a simplified isolation method for antigens. The use of antigens to isolate antibodies has been employed by immunologists for many years [1, 2]. The early use of immunoadsorbents to isolate antibodies was not always successful because the physical adsorption used to fix the antigen permitted "leaking". However, with the various methods available today for covalently bonding to solid supports, this is no longer a serious problem.

The use of immobilized antibody to isolate antigens is rapidly becoming an accepted laboratory technique [3, 4]. However, attempts in the past, to isolate biologically active molecules such as enzymes have not been successful due to low yields, usually less than 25% [5].

On the other hand, the isolation of nonbiologically active substances seems to be quite adaptable to this process.

It was our purpose to determine whether immunoadsorption could be employed as a simplified enzyme isolation method.

ISOLATION OF HUMAN PLACENTAL ALKALINE PHOSPHATASE

Human placental alkaline phosphatase was isolated from a crude suspension in a two-step immunoadsorbent procedure. The placenta had first been extracted with butanol and subjected to an ammonium sulfate fractionation to remove some of the extraneous protein. The immunoadsorbent in this case was agarose (Sepharose 2B) with rabbit serum immunoglobulin covalently bonded through the CNBr method [6].

A batch reaction gently mixing the immunoadsorbent with the crude enzyme solution was employed to effect the antibody-enzyme complex. It was felt that this is easier to control than a column and more acceptable to a large-scale system. After a sufficient reaction time the immunoadsorbent with the complexed enzyme was separated from the suspension and washed to remove nonspecifically adsorbed protein. Placental alkaline phosphatase was dissociated from the immunoadsorbent using 0.2 M Na_2CO_3, pH 11.4 [7].

In this case the usual agents for dissociated antibody-antigen complexes (e.g., KI, extremes of pH, KCNS) do not result in an active enzyme.

Yield and purification depend to a large extent on the affinity of the antibody for the antigen. A low affinity antibody permits greater dissociation. The greater the concentration of crude antigen exposed to the immunoadsorbent, the greater the degree of purification and yield (see Table 1). Immunoadsorbent 7 is made from serum drawn 7 weeks after initial inoculation, and immunoadsorbent 64 was drawn after 64 weeks. The pH at which crude antigen is associated with the immunoadsorbent also influences to a lesser extent how strongly the antigen is complexed. It was possible by using an anti-alkaline phosphatase immunoadsorbent to effect a purification of up to 100-fold [8].

Antibody to the alkaline phosphatase impurity, which in this case is human plasma, was also produced. By using a second

immunoadsorbent against the impurity, it is possible to effect another 2-fold purification. Thus in a two-step procedure, a 200-fold purification with about a 90% yield is obtained.

CARBOXYPEPTIDASE G-1

Carboxypeptidase G-1 also has been successfully isolated by immunoadsorption [9]. The immunoadsorbent and antigen are complexed at pH 6.5 and the complex dissociated by 4 M KCNS or by 6 M guanidine HCl (see Tables 2 and 3). The yield from this procedure was about 85% with a 35-fold purification. It is possible to account for about 95% of the activity entering the immunoadsorption process.

Antibody against the impurities was also produced and made into an immunoadsorbent for removing impurity from the partially purified carboxypeptidase. It was possible to effect another 1.5- to 2-fold purification doing this (see Table 4). The effectiveness of using an anti-impurity immunoadsorbent is limited because of the nonspecific adsorption of the enzyme which is in relatively high concentration as compared with the complexed impurities.

The antigen used to produce the carboxypeptidase antibody in this case was not pure. This probably accounts for reduced purification by immunoadsorbent.

With conventional purification procedures (about 10 steps with a final substrate affinity step which is delicate), it is possible to obtain an enzyme with a specific activity of 700. Using the two-step immunoadsorbent procedure, a specific activity of 225 was obtained. It is expected that with a purer antibody, a higher purification of enzyme would result from the first immunoadsorbent step.

REMOVAL OF SERUM HEPATITIS ANTIGEN FROM BLOOD PLASMA BY IMMUNOADSORPTION

It has been possible by immunoadsorption to remove serum hepatitis antigen from blood and blood plasma. Our initial work employed goat antibody to serum hepatitis antigen. The

TABLE 1

Influence of pH, Antigen Load, and Antibody Date on Yield and Purification of Placental Alkaline Phosphatase

Immuno-adsorbent	pH of association reaction	Tube no.	Ag Available S.A. 25 (units per mg Ig)	Ag associated (units per mg Ig)	Ag dissociated (units per mg Ig)	Percent Ag dissociated of total associated (%)	Specific activity of dissociated antigen	Degree of purification (fold)
7	7.4	1	2.7	2.6	1.6	61.7	61.3	24.4
		2	5.4	4.9	4.3	87.9	114.5	45.8
		3	10.7	8.9	6.6	74.9	134.6	53.8
		4	26.8	15.2	8.9	58.5	157.0	62.8
		5	48.2	21.1	11.0	52.2	169.8	68.0
		6	91.1	35.4	12.9	36.4	152.3	61.0
	8.6	1	2.7	2.3	1.1	46.7	18.9	7.5
		2	5.6	4.7	4.6	96.2	90.2	36.0
		3	11.3	7.9	7.4	94.5	119.5	48.0
		4	28.2	12.7	12.2	96.0	122.7	49.0
		5	50.7	15.1	15.0	99.3	112.2	45.0
		6	95.7	19.1	17.2	91.1	110.8	44.3
64	7.4	1	5.5	5.0	2.3	45.0	124.1	49.6
		2	11.1	8.8	3.3	37.6	162.3	64.9
		3	44.3	15.2	5.9	38.6	225.9	90.3
		4	60.9	18.9	6.7	35.8	259.4	103.7
		5	72.5	20.4	6.7	32.7	155.8	62.3

8.6	1	0.7	0.6	0.2	33.3	10.8	4.3
	2	2.1	1.8	0.7	37.3	40.6	16.2
	3	8.6	5.0	2.0	40.3	102.5	41.0
	4	13.6	4.5	2.0	44.1	73.0	29.2
	5	21.4	7.2	3.0	41.7	133.8	53.5
	6	51.3	13.8	4.2	30.7	180.3	72.1
	7	74.5	17.4	2.6	14.8	97.6	39.0

TABLE 2

Carboxypeptidase G-1 Activity and Protein Balances for Typical Batch Immunoadsorption and Elution Experiments using Guanidine HCl[a]

	INTO SYSTEM			OUT OF SYSTEM		
Run	Protein (mg)	Enzyme units	Specific activity		Enzyme activity (units)	Protein (mg)
#1	209.3	966	4.6	Primary filtrate and washes	568	201.4
				Amount of activity complexed with immunoadsorbent	378	———
				Dissociated	288	4.37
				% Recovery of complexed enzyme	72.4	
				Specific activity	167.4 μ/mg	
				Degree of purity	36.1	
#2	209.3	966	4.6	Primary filtrate and washes	620.7	197.7

				Amount of activity complexed with immunoadsorbent	345.3	———
				Dissociated	321.7	4.7
				% Recovery of complexed enzyme	93.2	
				Specific activity	156.0 μ/mg	
				Degree of purity	33.8	
#3	209.3	966	4.6	Primary filtrate and washes	599.3	189.9
				Amount of activity complexed with immunoadsorbent	366.7	———
				Dissociated	292.9	3.4
				% Recovery of complexed enzyme	79.9	
				Specific Activity	166.7 μ/mg	
				Degree of purity	36.1	

[a]Antibody #37. 9/26/73. Titer 1/16. Serum attached to Sepharose: 19 mg/cc. 5 cc samples about 0.95 g Sepharose/cc. Incubation time: 2 hrs. 3.6 cc of 6 M guanidine/g Sepharose for dissociations.

TABLE 3

Carboxypeptidase G-1 Activity and Protein Balances for Typical Batch Immunoadsorption and Elution Experiments Using 4M KSCN Followed by 6M Guanidine[a]

	INTO SYSTEM			OUT OF SYSTEM		
Run	Protein (mg)	Enzyme units	Specific activity		Enzyme activity (units)	Protein (mg)
#1	156	657.6	4.6	Primary filtrate and washes	149.3	139.1
				Amount of activity complexed with immunoadsorbent	508.3	———
				Dissociated enzyme		
				4 M KSCN	345.6	2.0
				6 M Guanidine	118.5	1.2
				Total recovery	464.1	
				% Recovery of enzyme associated	91.2	
				Specific activity		
				4 M KSCN	169.4 μ/mg	

				6 M Guanidine	98.8 μ/mg	
				Degree of purity		
				4 M KSCN	36.7	
				6 M Guanidine	21.4	
#2	156	657.6	4.6	Primary filtrate and washes	152.1	139.1
				Amount of activity complexed with immunoadsorbent	505.5	———
				Dissociated enzyme		
				4 M KSCN	310.5	1.7
				6 M Guanidine	147.6	1.3
				Total recovery	458.1	
				% Recovery of enzyme associated	69.6	
				Specific activity		
				4 M KSCN	184 μ/mg	
				6 M Guanidine	115 μ/mg	
				Degree of purity		
				4 M KSCN	39.1	

TABLE 3 (Continued)

				6 M Guanidine	24.9	
#3	156	657.6	4.6	Primary filtrate and washes	172	147.1
				Amount of activity complexed with immunoadsorbent	485.6	———
				Dissociated enzyme		
				4 M KSCN	284.2	1.7
				6 M Guanidine	135.3	1.3
				Total recovery	419.5	
				% Recovery of enzyme associated	86.42	
				Specific activity		
				4 M KSCN	165.0 μ/mg	
				6 M Guanidine	105.0 μ/mg	
				Degree of purity		
				4 M KSCN	35.7	
				6 M Guanidine	22.7	

#4	156	657.6	4.6	Primary filtrate and washes	148.6	137.5
				Amount of activity complexed with immunoadsorbent	509	———
				Dissociated enzyme		
				4 M KSCN	280.8	1.7
				6 M Guanidine	132.0	1.3
				Total recovery	412.8	
				% Recovery of enzyme associated	80.1	
				Specific activity		
				4 M KSCN	166.4 μ/mg	
				6 M Guanidine	105.0 μ/mg	
				Degree of purity		
				4 M KSCN	36.1	
				6 M Guanidine	22.7	

[a]Antibody #38. 7/13/73. Titer 1/32. IgG fraction attached to Sepharose, 19.89 mg/cc. 2cc samples. Incubation time: 2 hrs. Ratio of 10 ml elution solution/g Sepharose.

TABLE 4

Enzyme Activity and Protein Balances for Typical Batch Immunoadsorption of Contaminating Proteins

	INTO SYSTEM			OUT OF SYSTEM		
Run	Protein (mg)	Enzyme units	Specific activity		Enzyme activity (units)	Protein (mg)
#1	1.2	172.5	143.6 μ/mg	Primary filtrate and washes	172.8	0.780
				% Recovery of mass	65	
				% Recovery of activity	100	
				Specific activity	222.0	
				Increase in purity	1.55	
#2	1.2	172.5	143.6 μ/mg	Primary filtrate and washes	159.8	0.719
				% Recovery of mass	65	
				% Recovery of activity	93	
				Specific activity	222	
				Increase in purity	1.55	

immunoadsorbent may be prepared by attaching anti-immunoglobulin or antisera to Sepharose 2B by the CNBr method. It may also be made using glass beads or any other suitable support. The antigen is complexed by immunoadsorbent in a gently mixed batch system (see Fig. 1). Antigen is reduced by 90% each 2 hr. at 4°C. Thus in the first 2 hr., 90% is complexed; in 4 hr., 99%; in 6 hr., 99.9%; etc. At higher temperature the complexing rate is more rapid, e.g., at 25°C, 90% is complexed in 1.5 hr., and at 40°C in about 1 hr. (see Fig. 2). The immunoadsorbent-antigen complex may be dissociated with 0.23 M NH_4OH or pH 2.8 glycine-HCl and the immunoadsorbent reused (see Fig. 3).

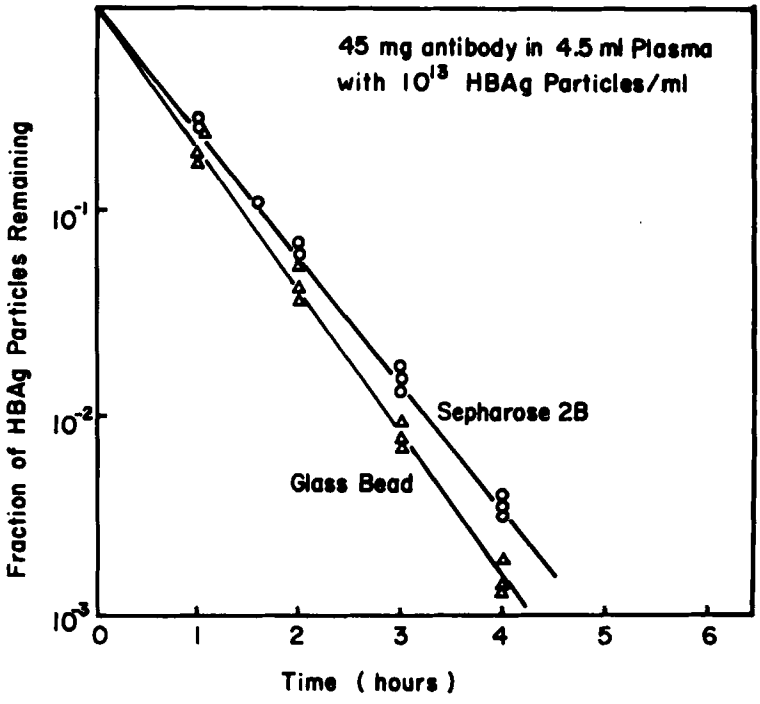

FIG. 1

Comparison of solid supports: Sepharose 2B gel and zirconia-clad glass beads.

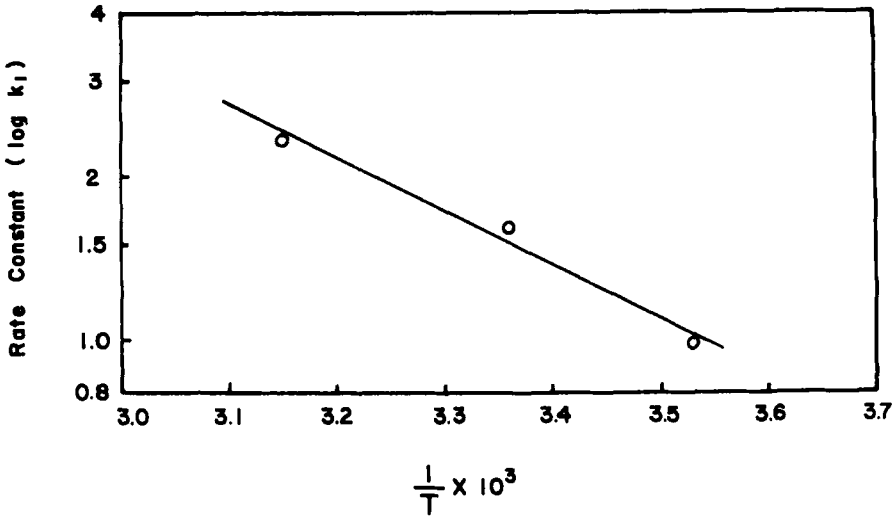

FIG. 2

The Arrhenius plot: activation energy of the HBAg immunoadsorption. Temperature ranges from 8 to 45°C.

With current detection methods, e.g. radioimmunoassay, it is possible to detect hepatitis antigen to levels of about 5×10^8 particles/ml. However, it is expected that 10^3 particles could result in disease [10].

In a general processing practice where only negative test material was employed, it may be assumed the initial concentration is not more than 5×10^8 particles/ml since this is the lowest concentration presently detectable by test.

In a 100-liter plasma pool there would be $5 \times 10^8 \times 10^5$ particles initially that should be reduced to a level where the probability is such that less than 1 antigen particle will remain in the pool. This would theoretically require about 27 hr. of mixing with the immunoadsorbent.

It must also be considered that equilibrium between the complexed and free antigen may occur at some point. Equilibrium was not reached over a 4 log cycle reduction (from 10^{13} to 10^9 particles/ml, the limit of detection). Therefore, regeneration of immunoadsorbent should be carried out after every

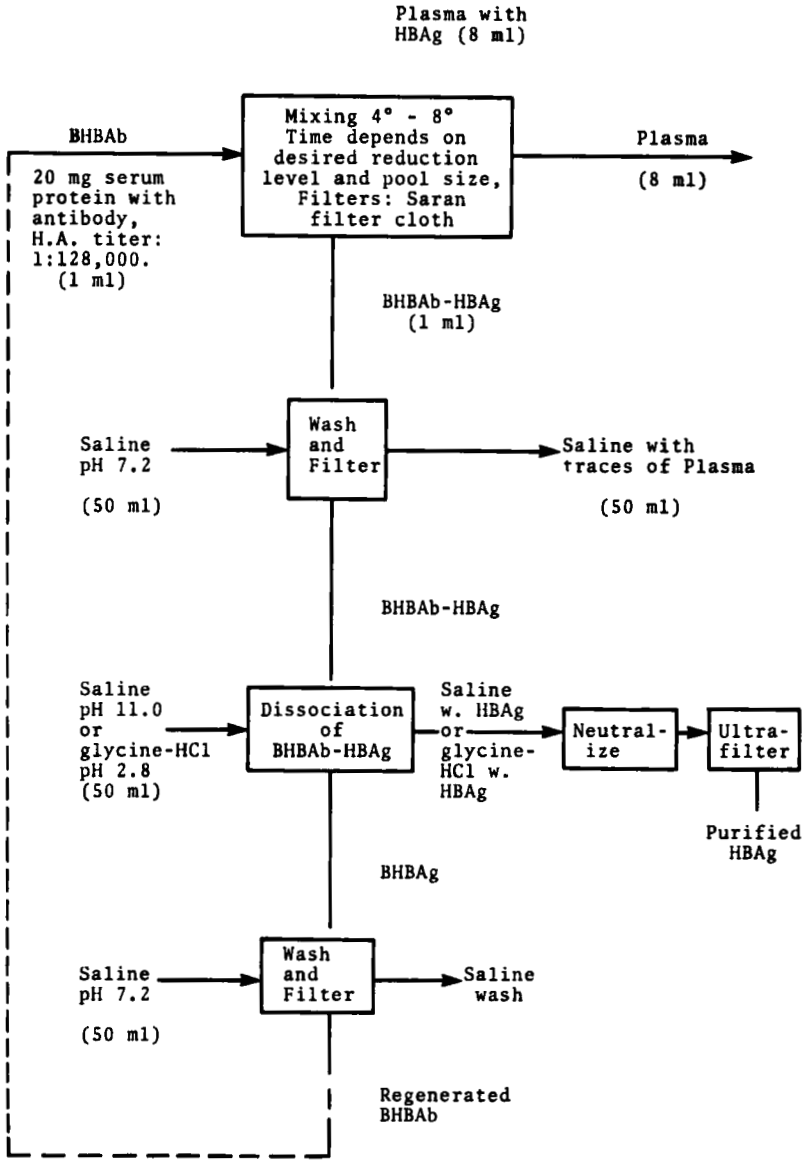

FIG. 3

Flow diagram of batch process for removing HBAg from plasma and recovering purified HBAg.

4 log cycle reduction to prevent equilibrium.

Increasing the concentration of antibody in the immunoadsorbent increases the rate of complexing up to a point. Then

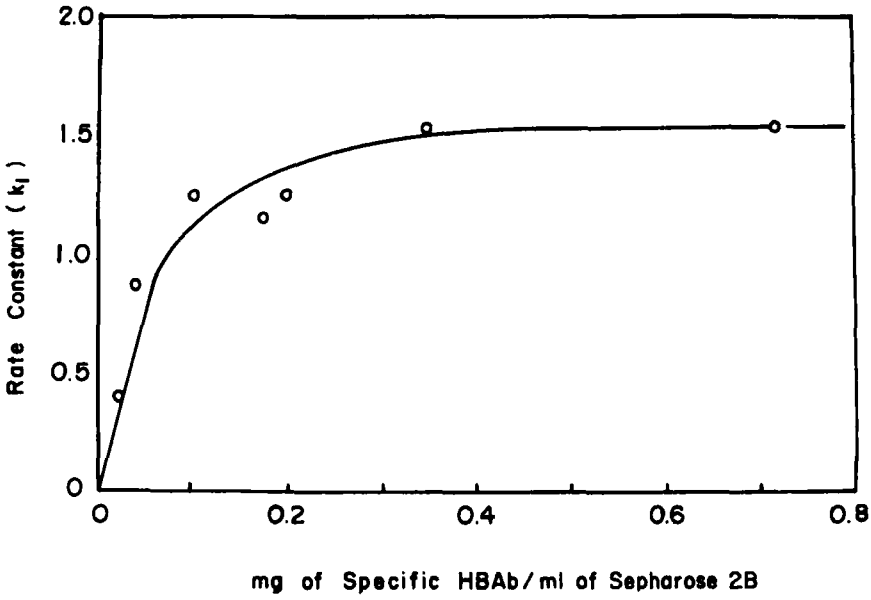

FIG. 4

Antibody concentration effect on the complexing rate.

other factors limit the complexing rate (see Fig. 4). Possibly the rate of diffusion of antigen to the immunoadsorbent becomes the limiting factor.

It has been possible to remove hepatitis infection from Factor IX (a plasma product with a high incidence of hepatitis associated) by this immunoadsorption process [10]. In this case a gibbon was used as the test animal. The gibbon was first inoculated with Factor IX that had been subjected to the immunoadsorption process. After 8 months, when it did not show any signs of infection, the gibbon was inoculated with unprocessed Factor IX. Within 14 weeks signs of hepatitis were observed. (This study was carried out by Dr. Hoofnagle, Department of Biologics, National Institutes of Health.)

Use of an immunoadsorbent made with animal antibody presents certain problems. Care must be taken that none of the animal protein leaks into the plasma. This could result in an

allergenic reaction. For this reason, human antibody obtained from hemophiliacs was tried.

The kinetics are similar but the affinity constant is much higher than that of animal antibody:

$$\text{affinity constant} = \frac{\text{free antigen at equilibium}}{\text{complexed antigen}}$$

The affinity constant from animal antibody is not known, but for human antibody it is about 10^{-2}.

Thus after using human antibody, the rate of complexing hepatitis antigen levels off after a reduction of about 2 log

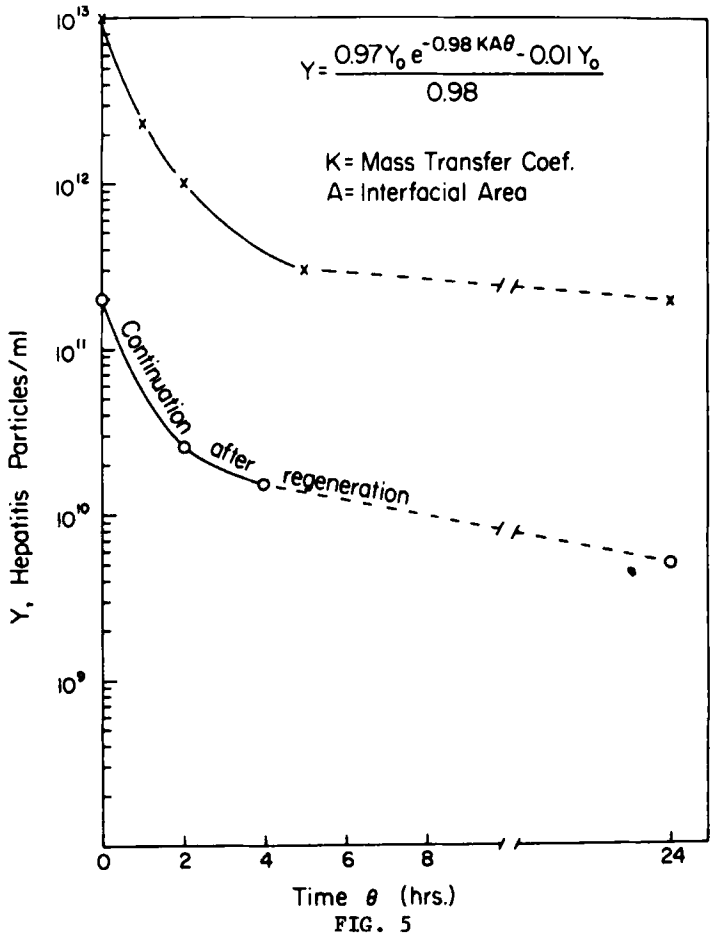

FIG. 5

Complexing of hepatitis antigen with human antibody immunoadsorbent.

cycles (see Fig. 5). For further reduction, the immunoadsorbent must be regenerated. Using human immunoadsorbent, it seems that plasma passage through an immunoadsorbent column would be more convenient that a batch adsorption of antigen that would require more regenerations of the immunoadsorbent to achieve the desired reduction of antigen than when using a higher affinity animal antibody. Serum hepatitis antigen recovered from infected plasma by this process is used to produce antibody in animals. The dissociated antigen is subjected to an antiplasma immunoadsorbent to remove traces of plasma. The final antigen is purified about 350X.

CONTINUOUS IMMUNOADSORPTION

A continuous immunoadsorption process has been developed using an endless belt of immunoadsorbent. The immunoadsorbent belt is driven through various compartments a) crude antigen → b) wash → c) dissociating solutions → d) wash → a) etc. (see Fig. 6).

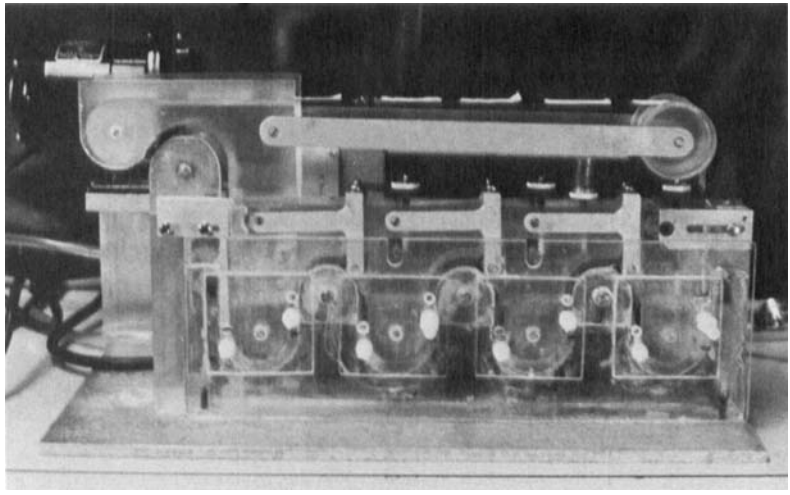

FIG. 6

Endless belt of immunoadsorbent for continuous antigen isolation.

The belt is made from high fiber bond paper protected by wide mesh fiberglass screening. The major problems associated with this system is the carry over from one compartment to another. This is overcome to some extent by employing "squeezers" between compartments.

This type of system could theoretically be employed for the continuous removal of serum hepatitis antigen from plasma using human antibody, avoid the equilibrium problem between complexed and free antigen associated with the batch processing.

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