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Isolation of the Heterogenetic "Mononucleosis Antigen" from the Stroma of Beef Erythrocytes.

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Stuart and coworkers[1,2] demonstrated the presence in beef erythrocytes of the heterogenetic "mononucleosis antigen" (M.A.) to which they gave the designation "B.T." ("beef thermostable antigen"). This antigen is contained in beef erythrocytes in a much greater concentration than in sheep erythrocytes. We have confirmed the findings of Stuart and coworkers, although we have not used the designation "B.T." since beef erythrocytes also contain other thermostable antigens which do not react with serum obtained from patients with infectious mononucleosis. These will be discussed in a later publication.

The previous method for determining serological activity consisted in measuring the decrease in the sheep cell hemagglutinin titer of a mononucleosis serum after adsorption by a thick suspension of erythrocytes. Our method, which we have already described in a previous communication,[3] is as follows: 1. Determination of the sheep cell agglutinin titer of the serum using the centrifugation method. 2. Adsorption of the serum by various dilutions of antigen in geometric series, in such a way that the final dilution of the serum corresponds to two agglutinin-units. 3. Removal of the insoluble material by centrifugation and testing the clear supernatant liquid for sheep cell agglutination.

Using this method, we are in a position to carry out activity measurements on our preparations and to specify the dilution in terms of dry substance.

We use as starting material the stroma obtained from hemolysed erythrocytes by centrifugation with the Sharples supercentrifuge.

The yield from 100 cc of defibrinated blood is about 0.5 g stroma. The activity varied on different days between 1:8000 and 1:32,000 for sheep stroma and between 1:32,000 and 1:128,000 for beef stroma, the variation being principally dependent on the sheep erythrocytes used for agglutination. On the other hand, the degree of activity is influenced little by the nature of the patient's serum, provided that the serum is a typical infectious mononucleosis serum and that 0.1 cc of the adsorption mixture prepared according to our technic contains 2 agglutinating units.

In a previous investigation,[4] we attempted to isolate the M.A. from sheep stroma. With pyridine and ethyl alcohol, etc., the Forssman antigen could be extracted while the M.A. remained behind in the stroma. Using N/30 NaOH or 1-2.5% $NaHCO_3$, it was possible to bring the M.A. with more or less success into solution, but without an increase in its serological activity.[3,4]

In this work beef stroma was studied since this offers a very much better source of heterogenetic M.A.

As Table I shows, the serological activity of beef stroma towards the heterogenetic infectious mononucleosis antibody is but very little decreased even after heating to 100°C.

Digestion with trypsin and pepsin was carried out in either a phosphate or a citric acid-phosphate isotonic buffer solution. The pH values were controlled potentiometrically. 1 mg enzyme was used for each cc stroma suspension (1:250 dilution). The activity of the enzymes was controlled by the fact that a suspension of fibrin flakes was completely digested within 2 hours, both at the beginning

[1] Stuart, C. A., Griffin, A. M., Fulton, Mc. D., and Anderson, E. G. E., PROC. SOC. EXP. BIOL. AND MED., 1936, **34**, 209.

[2] Stuart, C. A., Griffin, A. M., Wheeler, K. M., and Battey, S., PROC. SOC. EXP. BIOL. AND MED., 1936, **34**, 212.

[3] Tomcsik, J., and Schwarzweiss, H., *Schweiz. Z. Path. und Bakt.*, 1947, **10**, 407.

[4] Schwarzweiss, H., and Tomcsik, J., *Schweiz. Z. Path. und Bakt.*, in press.

TABLE I.
Inhibition of Sheep Cell Agglutinins in Infectious Mononucleosis Serum by Beef Stroma Heated for 30 Minutes.

Temperature °C	Beef stroma dilutions			
	16,000	32,000	64,000	128,000
40	—	—	—	++++
60	—	—	—	++++
80	—	—	+	++++
100	—	—	++	++++

TABLE II.
Inhibition of Sheep Cell Agglutinins in Infectious Mononucleosis Serum by Beef Stroma Digested by Trypsin or Pepsin.

Treatment of the beef stroma		Beef stroma dilutions
Pepsin at pH 3.0	2 days 37°C	1:128,000
" " " "	3 " "	1:128,000
Control " " "	3 " "	1:128,000
Trypsin at pH 7.7	2 days 37°C	1:32,000
" " " "	5 " "	1:64,000
Control " " "	5 " "	1:64,000

TABLE III.
Inhibition of Sheep Cell Agglutinins in Infectious Mononucleosis Serum by Beef Stroma Extracted with Organic Solvents.

Extraction	Extracted substance in %	Inhibition in dilutions	
		Extracted stroma	Extract
Acetone, room temperature	8.65	1:64,000	0
Alcohol, " "	20.0	1:16,000	1: 250
Alcohol, after acetone extraction, room temperature	12.6	1:16,000	1: 125
Ether, boiling	14.3	1:64,000	1:8000
Acetone, "	15.8	1:64,000	0
Pyridine, 90°C, after acetone- and alcohol-extraction, at room temperature	4.9	1: 8,000	1:2000

and at the end of the experiment. From Table II it can be seen that neither trypsin nor pepsin digests the M.A. In view of these experiments, it appeared improbable that the M.A. of beef erythrocytes is a protein. Apart from the M.A., beef stroma also contains a rapidly digested protein-like antigen which will be reported in a later publication.

Attempts were made to extract the M.A. with cold and with boiling organic solvents. The extraction with cold liquids was carried out by stirring for several days, the extraction with boiling liquids by changing the solvent several times until the material was exhausted.

As can be seen from Table III, a small quantity of M.A. could be extracted by means of boiling ether or hot pyridine. However, since, contrary to the statements of Stuart, Griffin, Fulton, and Anderson,[1] the cold alcoholic extract also shows a small but definite activity, the possibilities of extraction by means of boiling ethyl alcohol were thoroughly investigated as described below. Beef stroma was extracted, first with acetone and then with cold 100% alcohol. On each occasion 50 cc solvent per g stroma were used and the suspension stirred for 2 days at room temperature.

In this way, we were able to remove about 20% almost inactive material. After this pretreatment with cold organic solvents the beef stroma was exhaustively extracted with boiling 100% alcohol under reflux. The solvent was changed several times and, in this way,

TABLE IV.

Inhibition of Sheep Cell Agglutinins in Infectious Mononucleosis Serum by Beef Stroma Extracted First with Acetone and Ethyl Alcohol at Room Temperature and Later with Boiling Ethyl Alcohol.

Extracted with	Fraction	Activity of the extract
Acetone, room temperature	0-4 days	0
Alcohol, " " 100%	0-4 "	1:125
Alcohol, boiling, 100%	0- 15 min.	1:32,000
" " "	15- 30 "	1:16,000
" " "	30- 60 "	1: 8,000
" " "	60-120 "	1: 4,000
Alcohol, boiling, 80%	0- 15 min.	1:1,000,000
" " "	15- 30 "	1: 250,000
" " "	30- 60 "	1: 500,000
" " "	60-120 "	1: 250,000
" " "	2-4 hr	1: 500,000
" " "	4-8 "	1: 32,000
Stroma residue		1:8,000

TABLE V.

Purification of a Less Active Beef Stroma Extract. (Initial dry weight 1.7 g).

Extraction	Extracted substance in %	Inhibition of Sheep cell agglutination
Boiling acetone, 175 cc 0-15 min.	21.0	0
" " " " 15-30 "		
" " " " 30-60 "		
Boiling 100% alcohol 85 cc 0- 15 min.	16.2	1:4,000
" " " " " 15- 30 "	6.75	1:8,000
" " " " " 30- 60 "	2.0	1:8,000
" " " " " 60-120 "	1.6	1:8,000
Boiling 80% alcohol 85 cc 0- 15 min.	28.0	1:1,000,000
" " " " " 15- 30 "	4.55	1: 320,000
" " " " " 30- 60 "	0.7	1: 480,000
" " " " " 60-120 "	0.95	1: 320,000
Residue	29.2	

various fractions were obtained. After completion of the exhaustive extraction with 100% alcohol, which removed about 0.3-0.7% dry substance calculated with reference to the stroma, extraction under reflux was performed with 80% alcohol. The fraction so obtained contained the M.A. in a dissolved form and in very much higher activity than in the stroma itself.

Table IV shows that, using 100% alcohol, the M.A. isolated has only poor activity, but that, when 80% alcohol is used, the activity jumps to 1,000,000. Likewise, a drop in the activity of the stroma residue is manifest. Altogether 5.1% dry substance can be extracted from the stroma, with 80% boiling alcohol. This value shows a certain variation dependent upon the stroma preparation and upon the conditions of the experiment.

From numerous experiments we obtained fractions of high activity (1:256,000-1:1,000,000) and low activity (below 1:256,000), which we sometimes combined.

Further purification of the two fractions was achieved by the following procedure:

The alcohol was distilled off under reduced pressure the residue evaporated to dryness on the water bath and then exhaustively extracted under reflux, first with acetone, changing the solvent several times, then with 100% alcohol and finally with 80% alcohol.

In this way, a small quantity of highly active substance could be recovered from the fraction with low activity, an indication of the

efficiency of the method.

Table V shows that an activity of 1:1,000,000 can be reached starting from a mixed fraction possessing an initial activity of about 1:250,000.

After standing for one day at 4°C, the 80% alcohol extract with an activity of 1:1,000,000 gave a precipitate which was removed by centrifugation. The precipitate showed an activity of 1:80,000 and the material remaining in the solution an activity of 1:2,400,000. The last fraction, which was obtained as a brownish red powder after evaporating off the alcohol, was designated by us as a purified heterogenetic "mononucleosis antigen." It may be mentioned that we have attempted to extract the M.A. more readily from the stroma by saponification of the latter with sodium alcoholate (50 mg Na in 50 cc alcohol) under reflux. We found that this resulted in inactivation of the antigen.

We have attempted a further purification by treating the aqueous and the alcoholic solutions with activated charcoal both in the cold and at the boiling point. The charcoal effected complete decolorization, but, at the same time, the active substance also disappeared from the alcoholic solution. It could be detected in a suspension of the charcoal at a dilution of 1:8000 but could no longer be recovered.

Furthermore, we investigated the question whether M.A. is a necessary constituent of beef erythrocytes or whether as Sohier, Jaulmes, and Tissier[5] presume, there are individual variations. Apart from numerous tests with mixed blood, from which we were always able to obtain the antigen, we have examined blood from three animals separately. In each case, we were able to obtain the antigen with correspondingly good activity. A further source of M.A. is sheep's blood stroma. From this, using the extraction technic described above, we were only able to achieve activities of the alcoholic extract up to 1:8000.

Stuart, Griffin, Wheeler, and Battey[2] were able to demonstrate the presence of M.A. in rabbit serum by means of the hemolysis inhibition technic. Using our adsorption technic, we have examined dried rabbit organs: the heart, liver, spleen and cerebellum showed an activity of 1:250, the kidneys as much as 1:500, the stomach, small intestine, lungs and cerebrum only 1:64, and muscle was inactive. The heart, liver, spleen and kidneys were combined and extracted according to the scheme described. The cold acetone and alcohol extracts, as well as the boiling 100% alcohol fractions, were inactive. The boiling 80% alcohol extract exhibited an activity of 1:500 after 15 minutes and this rose to 1:4000 after extracting for 2 hours. As claimed by Stuart and his coworkers, the reason why the rabbit does not produce an antibody against M.A. fraction of beef erythrocytes is probably that it contains this antigen in its own organs. Thus, the heterogenetic M.A. occurs in beef and sheep erythrocytes as well as in rabbit organs. On the other hand, no trace of M.A. could be detected in rabbit erythrocytes, in peptone, in pepsin, or in trypsin preparations.

Summary. 1. From the stroma of beef erythrocytes it was possible to extract with boiling 80% alcohol the so-called heterogenetic "mononucleosis antigen," which, after purification, inhibits the sheep cell agglutination of the infectious mononucleosis serum in a dilution of 1:2,400,000. It is a thermostable hapten and is not digested by pepsin or trypsin.

2. The "mononucleosis antigen" which occurs in sheep stroma cannot be extracted with good activity by means of the technic effective for beef cells.

3. A serologically similar heterogenetic antigen could also be detected in rabbit organs, although in small quantities. This fact makes it comprehensible why the corresponding antibody cannot be artificially produced in rabbits.

[5] Sohier, R., Jaulmes, Ch., and Tissier, M., *Ann. Inst. Pasteur*, 1945, **71**, 463.