CCLVIII. STABILISATION AND PURIFICATION OF SPECIFIC ANTI-CANCER BODIES.

By THOMAS LUMSDEN AND THOMAS FOTHERINGHAM MACRAE.

From Cancer Research Department, London Hospital.

(Received September 26th, 1934.)

It has been shown in previous papers by one of us [Lumsden, 1926; 1931; 1932] that when an animal has been inoculated with a heterologous tumour, specific anti-cancer bodies can be demonstrated in its serum. These antibodies are chromatolysins and rapidly kill cultured cancer cells. They may be obtained from the serum in a somewhat purified state by preparation of the euglobulin fraction. Thus, for example, when a sheep has been inoculated fortnightly for some months with fragments of Jensen's rat sarcoma, the euglobulin fraction prepared from its serum by dilution and acidification is highly toxic to tissue cultures of cancer cells. This euglobulin fraction will destroy cultures of any malignant growth; it is much less, if at all, toxic to cultures of normal organs. Mice bearing spontaneous tumours have been cured and rendered immune to further inoculation by treatment with this euglobulin fraction [Lumsden, 1932], so that these anti-cancer bodies are potent *in vivo* as well as *in vitro*.

Great difficulty and inconvenience were experienced in carrying out the experiments above referred to owing to the lability of the anti-cancer bodies in the euglobulin fraction. The following paper describes the results obtained in a series of experiments designed to overcome this lability and to purify further these cytotoxic antisera.

EXPERIMENTAL.

In these experiments the potency and specificity of the various preparations have been tested as previously described by one of us [Lumsden, 1931] by applying them, adequately complemented, to malignant and normal cells cultured in serum. It is possible to compare fairly accurately the number of anti-cancer bodies in different samples by studying the damage done to the cultures of malignant cells by varying dilutions of the test samples.

Stabilisation of anti-cancer bodies.

Many antisera retain their potency over long periods when they are rapidly dried and stored at low temperatures. As was to be expected, the euglobulin fraction containing anti-cancer bodies can be stabilised in this manner.

The euglobulin precipitated by dilution of an anti-Jensen's-rat-sarcoma (A.J.R.S.) serum with nine volumes of water and acidification to p_H 5.6 with $N/10$ acetic acid is centrifuged down. The thick paste of euglobulin is rapidly transferred into a shallow glass basin and spread evenly over it. The basin is now placed in a vacuum-desiccator over phosphorus pentoxide and is kept in the cold store for several hours. The dry euglobulin may then be scaled off from

the basin in the form of translucent flakes and stored in sealed tubes. When stored at room temperature, euglobulin dried in this manner retains about 50 % of its potency after 3 months. During 5 months in the cold store, little or no diminution in activity occurs. The euglobulin dried and stored in this way is soluble in normal saline, but it dissolves more slowly than freshly precipitated euglobulin; the solution is only slightly opalescent. Active precipitates obtained from anti-cancer sera by precipitation with alcohol at low temperatures may be similarly stabilised by drying.

Other methods of drying the euglobulin were investigated. The euglobulin, immediately after it was centrifuged, was washed rapidly in the centrifuge-tubes three times with acetone and three times with ether. The temperature was kept as low as possible throughout by using previously cooled solvents, and by placing the tubes in a freezing mixture between the washings. After the third ether washing, the tubes were transferred to a vacuum-desiccator which was evacuated. The euglobulin thus obtained was a fine white powder which did not dissolve completely in normal saline. The potency of the preparation was much diminished by this method of drying, and we therefore do not recommend it.

Further purification of anti-cancer bodies by fractioning the euglobulin.

As previously recorded [Lumsden, 1932] preliminary tests indicated that the anti-cancer bodies in serum existed mainly in the second half of the total euglobulin precipitate. The fractional precipitation of the anti-cancer bodies from A.J.R.S. sheep serum by dilution and acidification has now been more carefully studied.

5 ml. of A.J.R.S. sheep serum were diluted to 50 ml. with sterile distilled water. $N/10$ acetic acid was added drop by drop and the hydrogen ion concentration was controlled by removing a drop of the solution from time to time, and testing with a B.D.H. bromothymol blue capillator. When the hydrogen ion concentration had fallen to p_H 6.5, a considerable precipitate had formed and this was centrifuged down. The centrifugate was now acidified to p_H 6.4, when a further precipitate resulted. This was again removed by centrifuging. The fractional precipitation was continued until no further precipitate was obtained on the addition of more acid. The hydrogen ion concentration was controlled throughout with B.D.H. capillators (bromothymol blue and bromocresol purple). In all, six protein fractions were obtained, of which the first fraction was the bulkiest; the other fractions were similar to one another in bulk. All fractions were dissolved in normal serum and were tested on tissue cultures of J.R.S. and normal tissues. The results are given in Table I.

Table I.

Fraction	1	2	3	4	5	6
p_H range over which precipitated	Above p_H 6.5	p_H 6.5-6.4	p_H 6.4-6.2	p_H 6.2-6.1	p_H 6.1-5.9	p_H 5.9-5.5
Effect on T.C. of J.R.S.	No effect	Very slight damage	Rapid destruction	Rapid destruction	No effect	No effect
Effect on T.C. of normal tissues (heart and kidney)	No effect	No effect	No effect	No effect	No effect	No effect

In many further experiments, using eight different sheep sera, the euglobulin was precipitated in three fractions—above p_H 6.4, p_H 6.4-6.1 and p_H 6.1-5.6. It was invariably found that the middle fraction contained nearly all the anti-

cancer bodies, although occasionally the first and third fractions were not completely inactive. At least 80 % of the total activity always existed in the middle fraction.

The weights of the three fractions were determined in two experiments after drying each fraction over phosphorus pentoxide *in vacuo*. The results are given in Table II. The sera used in these experiments were from different sheep. In each experiment 5 ml. of serum were used.

Table II.

Fraction	p_H range	Weight of precipitates mg.	
		Exp. 1	Exp. 2
1	Above 6.4	21.2	16.2
2	6.4-6.1	12.4	13.3
3	6.1-5.6	9.0	9.2

The exact hydrogen ion concentration at which the anti-cancer bodies contained in a particular antiserum come down varies to some extent with the species in which the antiserum has been prepared. Thus the anti-cancer bodies in an anti-mouse-carcinoma-63 serum prepared in a rat (A.M. 63 rat serum) are precipitated at a slightly higher hydrogen ion concentration than those in A.J.R.S. sheep serum. The fractions in the case of A.M. 63 rat serum comparable with those mentioned above for sheep antiserum are precipitated as follows. The first fraction is precipitated above p_H 6.6, the second fraction between p_H 6.6 and 6.2 and the third fraction below p_H 6.2. The second fraction contains the bulk of the anti-cancer bodies, the third fraction is slightly active and the first fraction contains no demonstrable anti-cancer bodies.

Precipitation of anti-cancer bodies from anti-cancer sera by organic precipitants.

Since acetone and alcohol have been largely used in the preparation of active concentrates from immune sera, we investigated their effects with the following results.

(a) *Acetone*. 2 ml. of A.J.R.S. sheep serum were diluted with 2 ml. of water. The mixture was cooled to 0° and 1 ml. of cooled acetone was added (final concentration of acetone, 20 %). The precipitated protein was centrifuged down and rapidly dried in the cold over phosphorus pentoxide in a vacuum-desiccator. Tested in the usual way, this protein fraction was found to contain only slight anti-malignant cell properties. Other experiments using 24 and 28 % acetone gave protein precipitates which were also only slightly active.

(b) *Alcohol*. 2 ml. of A.J.R.S. sheep serum were mixed with 2 ml. of water, and the mixture was cooled to 0°. 0.44 ml. of absolute alcohol cooled to 0° was added to bring the concentration of alcohol in the mixture to 10 %. The precipitated protein was rapidly centrifuged down, care being taken to keep the temperature as low as possible. The precipitate was rapidly dried in a vacuum-desiccator over phosphorus pentoxide. The centrifugate from the above was again cooled to 0°, and absolute alcohol was added to bring the concentration of alcohol to 12 %. The precipitate was centrifuged and dried as before. Further protein fractions were obtained by increasing the concentration of alcohol to 14, 16 and finally to 20 %. The anti-cancer potencies of the various fractions were determined. Two further experiments were carried out in which the concentrations of alcohol were directly brought to 16 and 20 % respectively (Table III).

Table III.

Fraction	1	2	3	4	5	—	—
% alcohol over which precipitated	0-10	10-12	12-14	14-16	16-20	0-16	0-20
Effect on T.C. of J.R.S.	Rapidly destroyed	Rapidly destroyed	Very little effect	No effect	No effect	Rapidly destroyed	Rapidly destroyed

The anti-cancer bodies are therefore precipitated by low concentrations of alcohol. Precipitation is almost complete at 12 % alcohol. The proteins precipitated above this concentration contain few, if any, anti-cancer bodies.

It has been difficult to carry out these experiments because of the necessity of keeping the temperature low during centrifuging. The solubility of the serum-proteins in dilute alcohol has a very high temperature coefficient and rapid re-solution of the precipitated proteins is apt to occur. In the above experiments this was prevented as far as possible by filling the centrifuge-cups with crushed ice.

Other methods of obtaining anti-cancer bodies from A.J.R.S. sheep serum.

A.J.R.S. sheep serum gives no protein fraction corresponding to the fraction obtained by Felton from anti-pneumococcal horse sera [1928], but the following modification of the methods used by Felton for the purification of the anti-pneumococcal bodies gave an active preparation from A.J.R.S. sheep serum.

5 ml. of A.J.R.S. sheep serum and 1 ml. of $M/10 \text{ Na}_2\text{HPO}_4$ were mixed and cooled to 0° . 1 ml. of absolute alcohol was added and the precipitated protein was centrifuged down at 0° . This was dissolved in 6 ml. of $M/40 \text{ NaCl}$ and adjusted to $p_{\text{H}} 4.5$ with $N/10$ acetic acid. As the acid was added, protein was precipitated, which partially redissolved at $p_{\text{H}} 4.5$. The undissolved protein was centrifuged off and found inactive. The centrifugate was adjusted to $p_{\text{H}} 5.8$ with $N/10 \text{ NaOH}$ and the precipitated protein was centrifuged down. This precipitate contained about 50 % of the anti-cancer bodies originally present in the serum.

The following method has also given active anti-cancer fractions from A.J.R.S. sheep serum. The total euglobulin prepared by dilution and acidification from 5 ml. of serum was dissolved in 5 ml. of normal saline and diluted to 50 ml. with water. The hydrogen ion concentration was adjusted to $p_{\text{H}} 4.5$ by the addition of $N/10$ acetic acid, and an inactive protein precipitate was centrifuged off. The supernatant liquid was adjusted to $p_{\text{H}} 5.8$ and was centrifuged; the resulting precipitate contained the bulk of the anti-cancer bodies existing in the total euglobulin.

These two methods for obtaining anti-cancer bodies from A.J.R.S. sheep serum probably give the same protein fraction as is obtained by selection of the middle euglobulin fraction precipitated by dilution and acidification. The methods are more tedious and the loss of anti-cancer bodies is greater.

Adsorption of anti-cancer bodies by aluminium hydroxide.

Eisler and Spiegel-Adolf [1929] have studied the purification of antibodies by adsorption on aluminium hydroxide followed by elution with $N/100 \text{ NaOH}$. They obtained considerable purification of typhoid agglutinin by this method. The adsorption and elution of serum-proteins have been investigated by Spiegel-Adolf [1932]. She found that euglobulin was readily adsorbed by aluminium hydroxide, but could not be quantitatively eluted by her methods.

With a view to obtaining further purification of the anti-cancer bodies from A.J.R.S. sheep serum, the adsorption and elution of these antibodies from the already purified preparation obtained by fractional precipitation of the euglobulin have been studied. The experimental conditions have been widely varied, but only partial success has been obtained. The antibodies were very readily adsorbed by aluminium hydroxide but it was not found possible to elute more than 40–50 %. The weight of eluted protein was about 30 % of the original weight, so that a slight gain in the antibody-protein ratio had resulted. The great loss of anti-cancer bodies in the process, and the small gain in the antibody-protein ratio render this method of purification of little practical value.

The following method was found to give the best results. The middle fraction of the euglobulin prepared from 5 ml. of A.J.R.S. sheep serum by dilution and acidification was dissolved in 2 ml. of normal saline. 0.5 ml. of *M*/5 sodium acetate-acetic acid buffer at p_H 4.5 was added, and the small amount of protein which was precipitated was centrifuged off; this precipitate contained no anti-cancer bodies. To the centrifugate 1 ml. of a suspension of aluminium hydroxide C_7 (15.6 mg. of Al_2O_3 per ml.) prepared according to Willstätter *et al.* [1925] was added. After shaking for 3 minutes the aluminium hydroxide was centrifuged down. The centrifugate contained no anti-cancer bodies; it contained however some coagulable protein which was isolated by heating to boiling and centrifuging off the coagulate. The aluminium hydroxide was now suspended in 2 ml. of *M*/10 phosphate buffer at p_H 7.5, and after 10 minutes' shaking, the suspension was centrifuged. The eluate was diluted to 25 ml. with water and adjusted to p_H 5.6 with *N*/10 acetic acid, when a protein precipitate was obtained. This was centrifuged down and the anti-cancer bodies determined. Many experiments were carried out, and in all cases some 40–50 % of the antibodies contained in the original euglobulin fraction were found in the protein isolated from the eluate.

Three experiments were carried out in order to determine quantitatively the adsorption and elution of the proteins. Each protein fraction was dried in a vacuum over phosphorus pentoxide (see Table IV).

Table IV.

	Exp. 1	Exp. 2	Exp. 3
Weight of original euglobulin mg.	16.9	15.5	13.3
Weight of protein precipitated by adjusting to p_H 4.5 mg.	2.8	2.0	2.0
Weight of unadsorbed coagulable protein mg.	3.5	2.5	1.9
Weight of protein isolated from the eluate mg.	5.7	4.9	3.0

From the above table it may be deduced that a large proportion of the adsorbed protein is not eluted from the aluminium hydroxide. It is probable that the loss of anti-cancer bodies is due to their remaining adsorbed by the aluminium hydroxide.

SUMMARY.

1. Anti-cancer sera can be highly concentrated by fractional precipitation of the euglobulin and subsequent adsorption and elution.
2. These concentrates, originally highly labile, can be stabilised by rapid drying, so that they remain potent for several months.

3. The specific anti-cancer cytotoxic bodies can be precipitated by low concentrations of alcohol.

We wish to express our thanks to the British Empire Cancer Campaign for whole time grants, which have made it possible for us to carry out the above research.

REFERENCES.

- Eisler and Spiegel-Adolf (1929). *Biochem. Z.* **204**, 28.
Felton (1928). *J. Infect. Dis.* **43**, 543.
Lumsden (1926). *Lancet*, ii, 112.
—— (1931). *Amer. J. Cancer*, **15**, 563.
—— (1932). *J. Path. Bact.* **35**, 441.
Spiegel-Adolf (1932). *Biochem. Z.* **252**, 37.
Willstätter, Kraut and Erbacher (1925). *Ber. deutsch. chem. Ges.* **58**, 2448.