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CHANGES OCCURRING IN PLASMA AND SERUM ON STORAGE AND THEIR PHYSIOLOGICAL EFFECTS

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Stored human blood, plasma and serum are now used extensively in the treatment of a wide variety of clinical conditions. Various workers—Lewisohn & Rosenthal [1933], Hughes, Mudd & Strecker [1938], Diggs & Keith [1939], Elliott, Macfarlane & Vaughan [1939], Girones [1939], Jorda [1939], Knott & Koerner [1939], Leedham-Green [1939], Lehmann [1939], McClure [1939], Patterson [1939], Aylward, Mainwaring & Wilkinson [1940], Black [1940], Brown & Mollison [1940], Brennan [1940], Brewer, Maizels, Oliver & Vaughan [1940], Clegg & Dible [1940], Edwards & Davie [1940], Ravitsch [1940], Scudder, Bishop & Drew [1940], Stewart [1940], and others—have reported on the use of whole blood, plasma and serum in human transfusion, the general consensus of opinion being that reactions to transfusion are infrequent, very slight, and, except in case of whole blood, do not increase with time of storage of the transfused material. Strumia, Wagner & Monaghan [1940 *a*, *b*] report favourable results with fresh and preserved plasma, but find that serum may cause severe reactions.

Landois [1875] suggested the transfusion of serum instead of whole blood. He injected a large number of heterologous sera into rabbits and dogs, and found that only those sera which agglutinated or haemolysed erythrocytes were toxic to recipients. Weiss [1896] described severe reactions on injection of serum from all species of animals into rabbits. Brodie [1900] found that cats were very susceptible to injections both of autogenous and heterogenous serum, and described a reaction to these injections which has since been known as the 'Brodie phenomenon'. All the sera investigated were toxic to cats, the doses required to produce a reaction varying from 0·1 to 10 c.c. Cats' blood withdrawn, defibrinated and reinjected within 2 min., he found to be toxic. Other animals, such as dogs, Brodie found to be almost insusceptible to serum. These results of Brodie's were in a large measure confirmed by the work of Ponder [1928]. Ponder found, however, that only 80% of the sera which he investigated were toxic to cats, and of these, only 50% showed a

high degree of toxicity. Ponder gives no information as to the ages of the various sera which he used. Doerr [1910] noted the similarity between anaphylactic shock and the toxic reactions to the injection of normal blood and serum. Zinsser [1911] described a toxic property of normal blood which was independent of its lytic and agglutinative properties. De Kruif [1917] and Novy & De Kruif [1917] found that fresh serum, injected as soon as possible after coagulation, was more toxic than old serum. They reported that plasma was non-toxic, but became toxic in the 'pre-clot' condition. The anaphylactoid shock produced by the injections was attributed to the production of an 'anaphylatoxin' in the animal's blood, in response to the injection of serum. Rous & Wilson [1918] evaluated the efficiency of the different fluids used as blood substitutes. They concluded that plasma was very good for this purpose, and that horse serum was effective in non-susceptible animals, e.g. dog and rabbit. Plasma and serum were more effective in restoring the blood pressure after haemorrhage than was Bayliss's gum-acacia solution. Bayliss [1920] investigated the reactions to transfusion, and suggested that the results of transfusion of incompatible blood were not due to haemolysis as such, but to a reaction to foreign serum protein, and that they were comparable to anaphylactic shock. Drinker & Brittingham [1919], reporting on the use of autogenous plasma and serum in transfusions, said that plasma which had been thoroughly freed from cellular elements by centrifugation or by filtration through porcelain was singularly non-toxic. In this respect, plasma differed markedly from serum. Levinson [1923] described an increased toxicity of plasma in pregnancy and in certain pathological conditions; this he attributed to an increase in the fibrinogen content of the plasma. Bond & Wright [1938] gave transfusions of lyophile serum to severely shocked dogs and found that this raised the blood pressure for several hours, but did not give recovery as the degree of shock was too great. Wright, Bond & Hughes [1938] used four times concentrated serum in doses of 4 c.c./kg. in order to reduce the cerebro-spinal fluid pressure in dogs. They found that the concentrated serum was effective for this purpose, and record no untoward results of the injections. However, Levinson, Neuwelt & Necheles [1940] reported severe reactions in 25% of dogs which they transfused with serum. Best & Solandt [1940], and Magladery, Solandt & Best [1940], found serum and plasma to be equally effective in restoring the blood volume of dogs shocked by histamine, trauma, trauma and haemorrhage, or haemorrhage alone. They took the precaution, however, of testing the sera to be used on the animals before shock was produced. Some samples of pooled dog serum did cause reactions. These were discarded and were not used for the attempted restoration of the shocked animals. Fine & Gendel [1940] gave dilute plasma (1–3 days old) to dogs with experimental intestinal obstruction. The survival time of these dogs was doubled when plasma was given in sufficient quantity

to compensate for the loss of fluid from the circulation. Buttle, Kekwick & Schweitzer [1940] found fresh plasma to be a good blood substitute in the treatment of acute haemorrhage in cats; no stored fluid plasma was used, but reconstituted plasma which had been dried by Dr Greaves proved fairly good. Plasma dried by Dr Davie killed the animal. When serum was used, severe reactions occurred in some cases. The authors noted the similarity between these reactions to serum and the Brodie phenomenon. They tried the effect of serum on dogs (which Brodie stated to be insusceptible) and noted a similar reaction in three cases. Reid & Bick [1942], investigating the pharmacological properties of serum, state that cats (but not dogs or rabbits) give severe reactions to the intravenous injection of homologous serum. They consider that the serum constituent responsible for this reaction is protein in nature, and is derived from the platelets.

We have not found any reference to work directed towards changes occurring in plasma or serum on storing, or the physiological effects of such storage. The work described below was undertaken when it was found that plasma or serum which had been stored 3–4 weeks in the blood bank was highly toxic to cats when injected intravenously in doses of from 0·5 to 2 c.c. whereas fresh human plasma or serum had no effect. Similarly, as reported below, fresh cat plasma is innocuous, whereas the same plasma becomes toxic on storage.

Methods

Cats were anaesthetized with liquid 'Dial' usually injected intraperitoneally (0·6 c.c./kg.), but when intestinal plethysmography was attempted the anaesthetic was injected subcutaneously (0·7 c.c./kg.). Some cats were anaesthetized with chloralose or nembutal to rule out the effect of a particular anaesthetic; a few decerebrate preparations, decerebrated under ether anaesthesia 2–3 hr. prior to the experiment, were also used.

The trachea was cannulated and systemic arterial blood pressure was recorded from the carotid artery. In some cases pulmonary arterial pressure, and in some venous pressure (either from the jugular or splenic vein) was also recorded. Intrapleural pressures were recorded in some animals by means of a pleural cannula [Franklin & Gilding, 1932], and records were also obtained from intestinal and limb plethysmographs.

The human serum and plasma used for injection into the animals were samples of pooled plasma obtained through the agency of Dr W. H. P. Cant (Regional Transfusion Officer, Midland Region, No. 9) from the Army Blood Supply Depot, Bristol, the Ministry of Health Regional Transfusion Laboratory, Birmingham, the Ministry of Health Regional Transfusion Laboratory, Oxford, the Medical Research Council Blood Depot, Social Centre, Slough, and the Medical Research Council Serum Unit, Cambridge. Except where otherwise stated, the plasma or serum was of varying age, from 4 weeks

to 18 months. Doubtfully sterile or unduly cloudy samples were discarded. The cat plasma or serum was obtained and stored under sterile conditions. The serum or plasma was warmed before injection. The amount given was from 0·5 to 2 c.c. according to the size of the cat and the activity of the sample. Usually the injection was made into the internal saphenous vein in front of the ankle, to ensure thorough mixing of the plasma with the animal's blood, but some were made into the splenic vein or into the femoral artery.

Results

The results of injection of active plasma or serum into the saphenous vein and into the femoral artery were similar, except that the reaction took slightly longer to come on when the injection was made by the arterial route.

Heart and blood pressure and peripheral circulation. Fig. 1 shows the effect of plasma on the heart and systemic blood pressure. Within 15 sec. the heart slows profoundly and, in a sensitive animal, may cease to beat altogether. The blood pressure falls almost to zero; cardiac massage and artificial respiration may be necessary in order to revive the animal. In some cases, after the cardiac inhibition has passed off, there are frequent dropped beats, indicating injury to the heart muscle. These findings agree with those of Brodie [1900] and Ponder [1928]. Records of pulmonary arterial pressure show that this rises as a result of plasma injection, the beginning of the rise being coincident with the beginning of the fall in systemic pressure (Fig. 2). Systemic venous pressure rises from about $-4{\cdot}5$ to $+4$ cm. saline (Fig. 3). Portal venous pressure shows no significant change.

Limb volume increases after the injection, but the volume of the intestine decreases (Fig. 4). This diminution in volume depends on the mechanical emptying of the vessels as a consequence of the greatly increased peristalsis noted below.

Brodie described a vasodilatation in limbs and a vasoconstriction in the kidney as a result of serum injection.

Respiration. Respiration may cease altogether and artificial respiration may be necessary in order to revive the animal, or there may be spontaneous recovery. In the latter case, periods of apnoea are interposed between two or three gasps, and eventually normal respiration is resumed (see Fig. 5). Records of intrapleural pressure show a rise in the base line of the water manometer, indicating a decrease in the negative pressure in the thorax. This decrease in negative pressure is probably the result of an accumulation of blood in the thorax. Fig. 5, which is typical of all the intrapleural pressure records which were made, shows that for each inspiration after plasma or serum injection a greater negative pressure is required. This increase in negative pressure is doubtless the result of bronchial constriction, since the injection of adrenalin brings about an immediate reduction in the negative

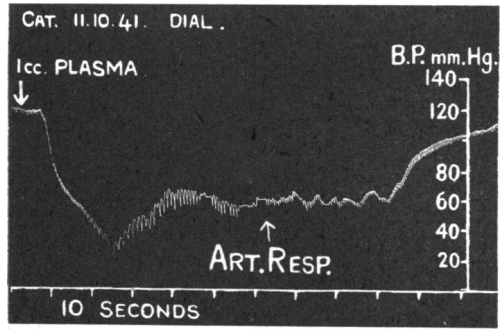

Fig. 1. Effect of 1 c.c. stored plasma on heart rate and arterial pressure.

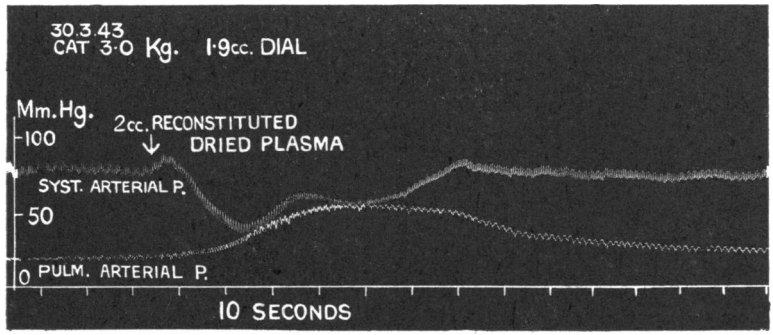

Fig. 2. Effect of 2 c.c. reconstituted dried plasma on heart, systemic and pulmonary arterial pressures. Normal respiration.

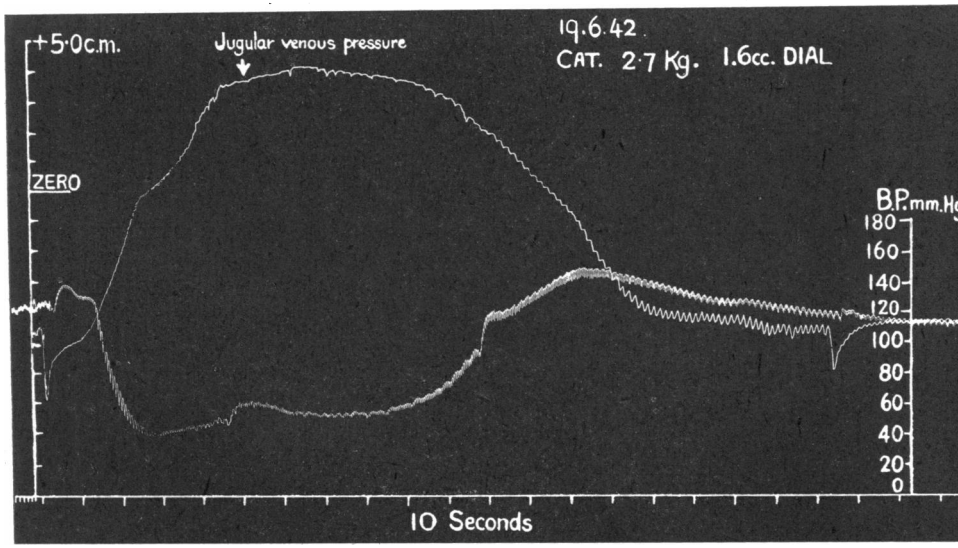

Fig. 3. Effect of 2 c.c. stored plasma on jugular venous pressure and systemic arterial pressure.

pressure required for inspiration. The effect of the adrenaline, however, soon wears off. The variations in intrapleural pressures do not return to normal

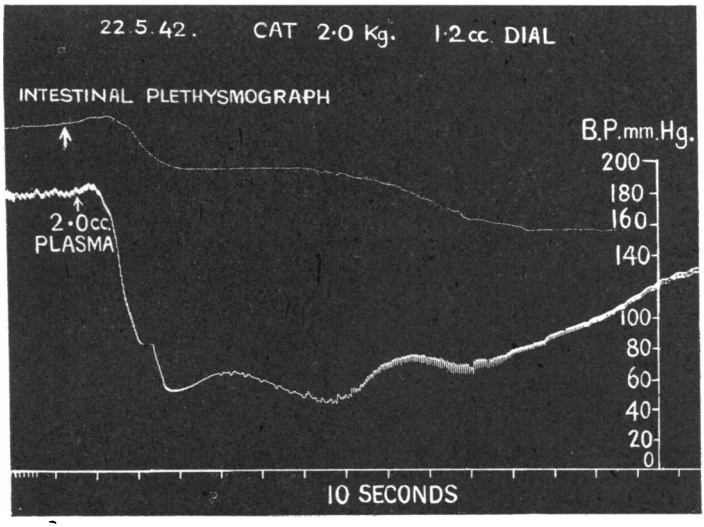

Fig. 4. Effect of 2 c.c. stored plasma on intestinal volume and systemic arterial pressure.

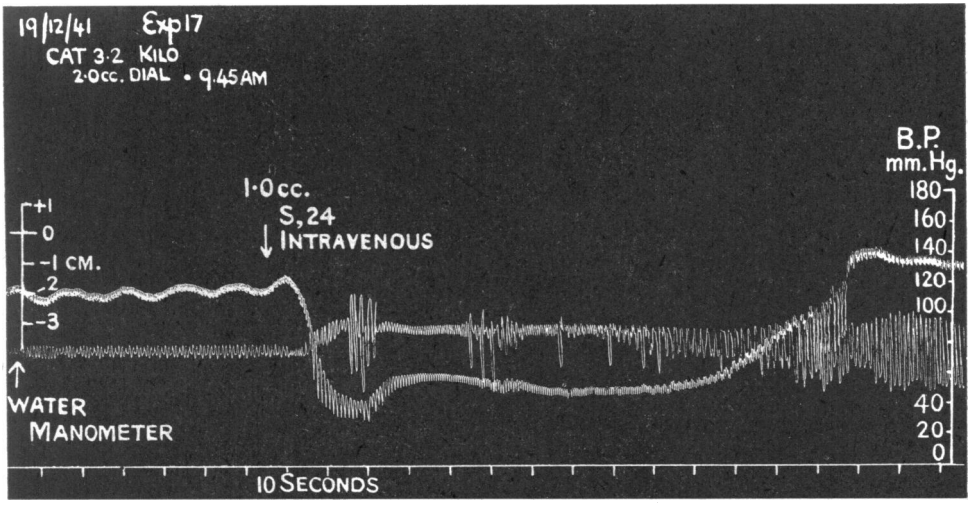

Fig. 5. Effect of 1 c.c. of stored serum on intrapleural and systemic arterial pressures. The oscillation of manometer during apnoeic periods is due to transmitted heart pulsations.

until a period of 10–20 min. has elapsed. In some cases rapid shallow respirations precede the complete cessation of respiration.

Alimentary canal. The intestines, examined either through an abdominal wound, or in the intestinal plethysmograph, show greatly increased peristalsis.

Animals with intact abdomens produce noticeable borborygmi. Retching may occur, and if the animal has been fed, vomiting. In decerebrate animals defaecation occurs.

Bladder. Micturition occurs if urine is present.

Eyes. Within 10–15 sec. of the injection, the nictitating membrane retracts, the palpebral fissure widens and the pupils dilate slightly. The dilatation is immediately followed by an intense pupillary constriction, the pupil being reduced to a mere line. This condition lasts for from 2 to 5 min., when the nictitating membrane, palpebral fissure and pupil return to the pre-injection condition. Usually these signs are accompanied by the secretion of tears. In some animals the constriction of the pupil is immediate and is not preceded by dilatation.

Saliva. Obvious salivation has never been observed.

Sweating. There were no signs of sweat on the animals' pads.

Movements. In anaesthetized animals there was marked opisthotonos, flattening of the pinnae and stretching movements of the limbs. In decerebrate animals, in addition to opisthotonos, the limbs performed co-ordinated rapid running movements for a period of about 2 min. after the injection.

Autopsy. The most striking pathological changes were seen in the lungs, where there were petechial haemorrhages and occasional areas of congestion. Portions of the lung were sectioned, and showed in some parts extravasation of blood into the connective tissue, and, in isolated patches, rupture of the alveolar walls with haemorrhage into the alveoli. The pulmonary vessels, particularly arterioles and capillaries throughout the lungs, showed an abnormally large number of leucocytes of the polymorphonuclear type. That this finding was the result of injecting stored plasma or serum was proved by removing a lobe of lung, closing up the thorax by suture before giving plasma, and then removing another lobe at autopsy after injecting plasma. Examination of subsequent wax sections of these two lobes showed a normal appearance in the lobe removed before plasma injection, while the section of lung after injection showed the usual picture seen in Pl. 1, fig. 6. In experiments in which the animals were left up to 7 hr. before being killed, the lungs still contained abnormal numbers of polymorphonuclear cells inside vessels.

The suprarenals showed little macroscopic damage, but occasionally there were patches of haemorrhage in the cortex. Wax sections showed numerous polymorphonuclear cells in the small vessels, more particularly in the cortex. This effect was controlled by removing a suprarenal before injecting the plasma. Here, as in the lung, the accumulation of polymorphonuclear cells was shown to be the result of the plasma injection (see Pl. 2, figs. 7 *a*, *b*). The liver appeared normal but here again an increased number of leucocytes was seen in section. The accumulation of white cells was, however, not so marked as in the lung and suprarenal.

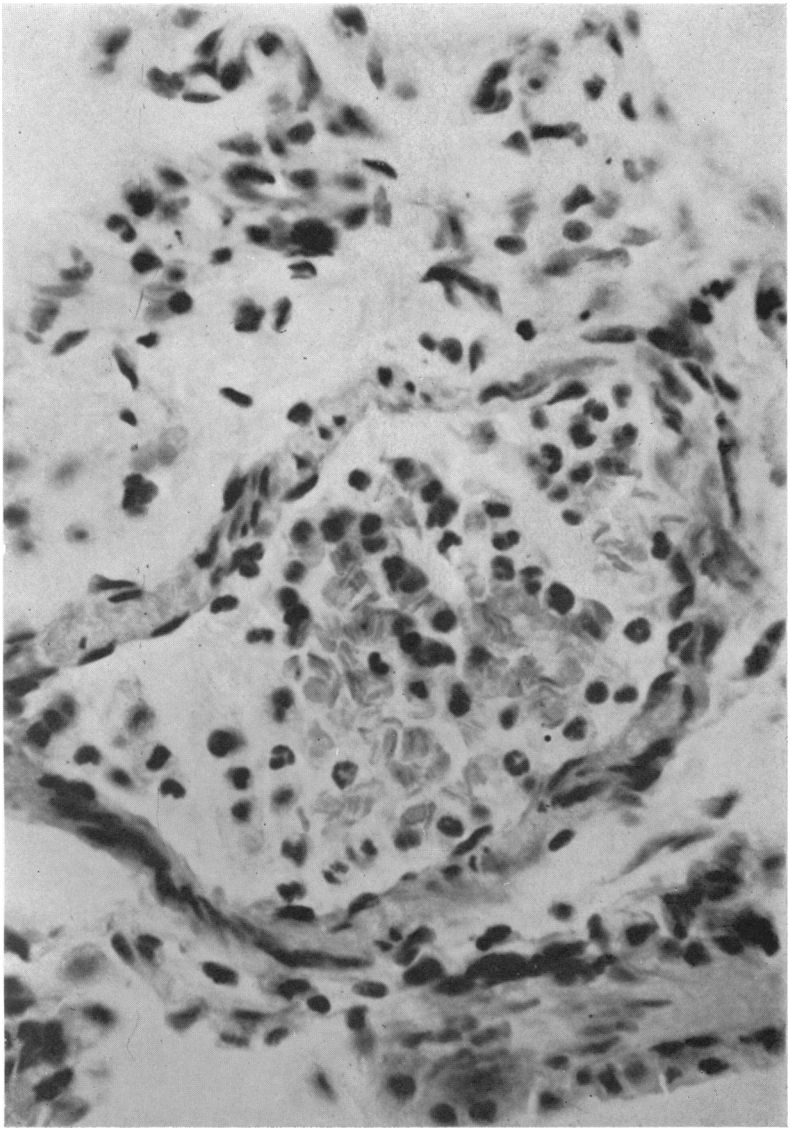

Fig. 6. Lung removed at autopsy after plasma, showing numerous polymorphonuclear leucocytes in lumen of arteriole. Wax section H. and E. ×520.

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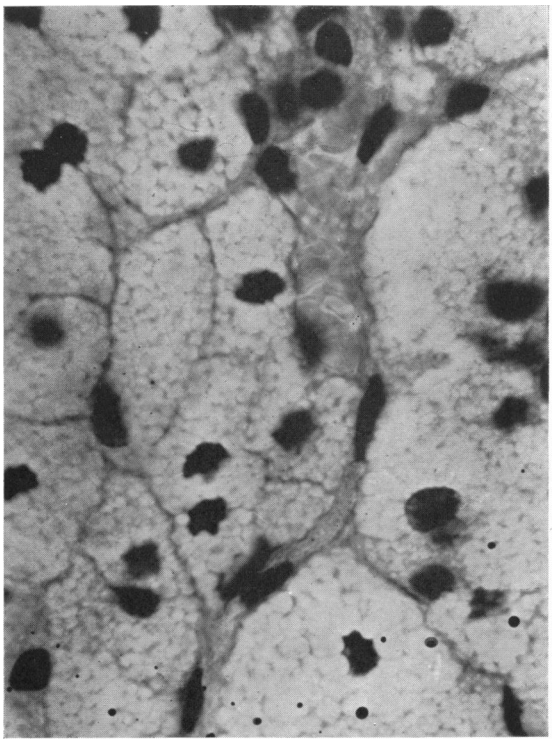

Fig. 7*a*. Wax section H. and E. of suprarenal removed before injection of plasma, showing capillary with many red blood cells but no leucocytes. × 1060.

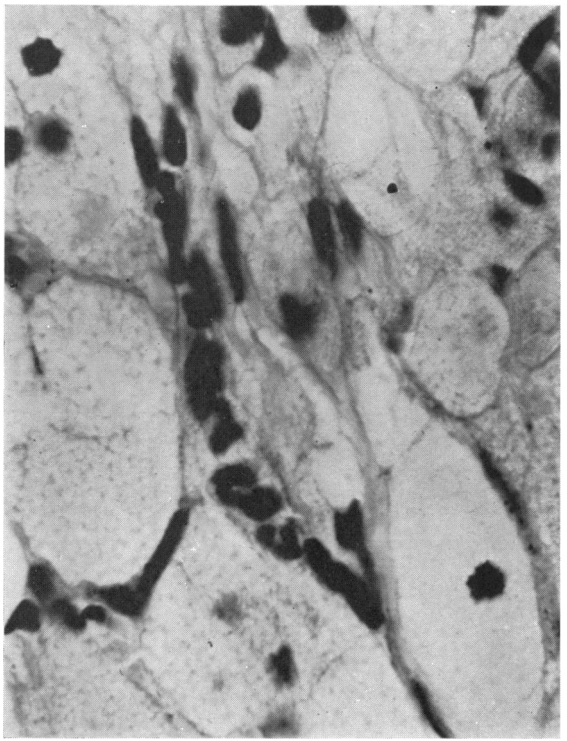

Fig. 7*b*. Wax section H. and E. of suprarenal removed at autopsy after plasma, showing capillary packed with polymorphonuclear leucocytes. × 1060.

Other tissues, such as spleen, kidney, intestine, skeletal muscle and diaphragm, showed no macroscopic or microscopic abnormalities.

Blood changes

The above histological findings raised the question of the leucocyte content of the blood before and after plasma injection. For this investigation a bleeding cannula was inserted into a femoral artery, and blood was taken before and after giving plasma. In order to correlate these findings with the other actions of plasma, samples of blood were usually taken between 30 and 40 sec. after giving the injection. Examination of the blood revealed a pronounced leucopenia, mainly, though not entirely, due to a diminution of polymorphonuclears. A protocol, which is typical of all experiments whether human or cat serum or plasma was injected, is as follows:

22 June 1942. Experiment 54. Cat 3·3 kg. 1·7 c.c. Dial intraperitoneally

	Before injection		After injecting 2 c.c. cat serum (collected 13 May 1942)	
Total white cells ...		6000		3100
Polymorphs	50%	3000	17%	527
Lymphocytes	37%	2220	66%	2046
Eosinophils	12%	720	13%	403
Basophils	1%	60	3%	93
Monocytes	—	—	1%	31
Haemoglobin	67%	—	62%	—
Haematocrit	37·6% (cells)	—	35%	—

After the initial leucopenia, the white cell count rose, and after 5–6 hr. was three or four times as high as the pre-injection level, the leucocytosis being mainly due to youthful polymorphonuclear cells; a number of nucleated red blood cells were seen, but no counts of these were made. The white cell count has not been followed for more than 7 hr. after plasma injection.

The injection of fresh human plasma had no action on leucocytes; the white blood cell count before injection was 8150 and after 7900, and the polymorphs 42 and 44% respectively.

The above experimental findings of the effect of stored plasma in the cat suggest: (*a*) stimulation of the parasympathetic nervous system either peripherally, centrally or reflexly; (*b*) stimulation of the central nervous system; or (*c*) a generalized chemical or physical action on the systems of the body. The following experiments were designed therefore to localize the site or sites of action of stored plasma.

Effect of atropine. Injection of plasma into the atropinized animal (atropinization tested with 5–10 μg. of acetylcholine) produced a fall in blood pressure, but no cardiac inhibition. The fall was not so marked as in the normal animal, and recovery was more rapid (Fig. 8). The effect on respiratory rate was similar to that in the non-atropinized animal, but there was no bronchial constriction, and the rise in the general baseline of the intrapleural pressure did not occur (Fig. 8). This suggests that the rise in intrapleural pressure

referred to earlier is the result of cardiac inhibition, which brings about an accumulation of blood in the thorax. The limb volume increased, and the intestinal volume—which in the non-atropinized animal diminished markedly —also increased slightly. This finding suggests that plasma may have a direct dilator action on blood vessels. After atropinization, plasma had no action on the intestinal muscle; no peristalsis was observed, neither defaecation nor vomiting, and there was no micturition. The pupils constricted slightly as a result of plasma, although acetylcholine had no effect on them. This constriction may possibly have been due to a central inhibition of sympathetic

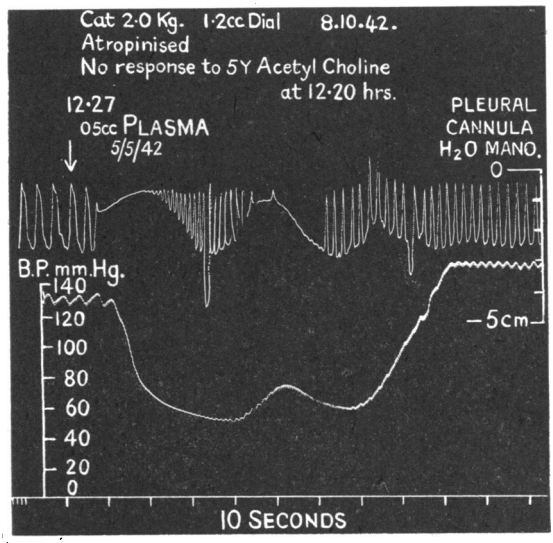

Fig. 8. Effect of 0·5 c.c. stored human plasma on heart rate, blood pressure and intrapleural pressure in the atropinized animal.

tonus. Opisthotonos and stretching of the limbs was diminished by atropine. The changes in the white blood cells were not influenced by atropinization of the animal.

Effect of section of the vagus nerves. Section of the vagus nerves in the neck abolished the cardiac inhibition, but there was still a fall in blood pressure, although this was less than when the vagi were intact. Fig. 9 shows the effect of cutting the vagi during the reaction. As far as respiration was concerned, section of the vagi abolished the changes in rate produced by plasma, but bronchial constriction was even more marked. Fig. 10 shows this effect, and shows also that there is no rise in the base line of the intrapleural pressure such as occurs with intact vagi. It also shows the effect of adrenaline on the bronchial constriction. Plethysmographs record an increase in limb and intestinal volume. The intestines no longer showed increased peristalsis. There

was no defaecation, vomiting or micturition and no opisthotonos or limb movements.

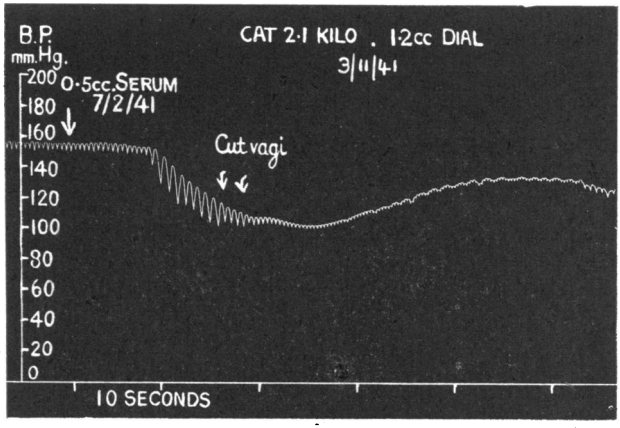

Fig. 9. Effect of vagal section on heart rate and blood pressure during response to injection of 0·5 c.c. of stored human serum.

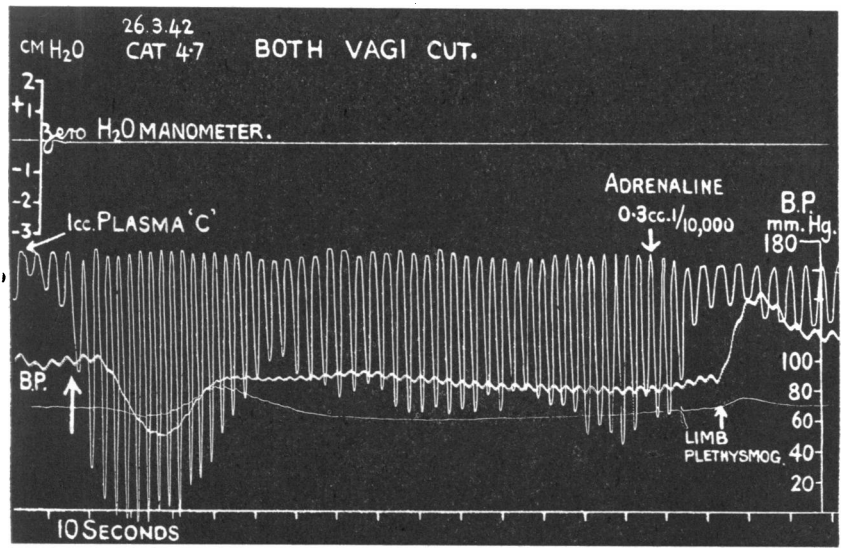

Fig. 10. Effect of 1 c.c. of stored human plasma on intrapleural pressure, heart rate, blood pressure and limb volume in vagotomized cat, and injection of adrenaline, 0·3 c.c. of 1/10,000 during the response.

The pupillary reactions were unaffected by vagal section. Section of the cervical sympathetic and splanchnic nerves in addition to the vagus abolished the reaction. The pupillary reactions may therefore depend in part on reflexes from the intestine. Probably the reactions are similar to those described by

Bain, Irving & McSwiney [1935] and by Irving, McSwiney & Suffolk [1937] as a result of stimulation of vagal and sympathetic nerves from the abdominal viscera.

Brodie [1900] showed that the site of action of serum was on afferent vagal endings in the lungs, and that the response to its injection was a reflex phenomenon. This he attempted to prove by cutting the vagal fibres running to the lungs, but, as he points out, the operative disturbance and subsequent necessary artificial respiration interfered considerably with the reaction. Brodie states, in fact, that the reaction cannot be obtained successfully from cats under artificial respiration, and that it is necessary to switch off the pump in order to observe it; we have not found this, probably on account of the different method used for ventilating the animal. Cats under artificial respiration show the vascular and cardiac reactions characteristic of plasma if the nervous system is in reasonable condition. However, as the phenomenon is largely a reflex one, cats which have suffered severe anoxia of the central nervous system as a result of respiratory failure will give very unreliable results.

Owing to the difficulties involved in section of the pulmonary vagus, we attempted to show whether the phenomenon was reflex or central in origin by means of cross-circulation experiments. The arterial connexion between carotid arteries presents little technical difficulty. The external jugular veins, however, constrict to such an extent that it is difficult to insert a cannula big enough to ensure an adequate venous drainage. This difficulty, however, can be overcome in part by exposing two areas on each external jugular vein, about 2 cm. apart, by snipping off the skin over the vein with scissors and leaving the exposed connective tissue and vein unprotected. The necessary ligatures are inserted deeply under the vein (which should not be dissected) by curved surgical needles, and left *in situ* until wanted. The exposed surfaces dry somewhat and, when the time comes to cannulate, the vessel does not contract to anything like the extent which it does without this precaution.

Two animals of approximately the same size were chosen, and both carotid arteries and external jugulars of each prepared for cannulation; the internal jugulars were tied. A strong cord was put in position around the vertebral column, at the level of the first and second cervical vertebrae, to occlude the vertebral arteries [Sherrington, 1919]. Then 17 mg. of heparin was injected intravenously into each animal. The animals were placed side by side and the neighbouring jugular veins cannulated proximally and distally and joined with rubber tubing, so that blood from each animal's head flowed into the other's heart. Next both carotids were joined in a similar manner. Then the outside jugular veins were similarly treated, and finally the vertebral arteries were tied off.

The blood pressure was recorded from the femoral artery in each animal. Fig. 11 shows the effect of an injection of 1 c.c. of stored human serum into an internal saphenous vein in cat A. It will be seen that the cardiac effects and fall of blood pressure occurred in the animal receiving the injection, in spite of the fact that its head was receiving blood from cat B's systemic circulation.

The efficiency of the cross circulation was tested by injecting 3 c.c./kg. of a 4% solution of bromphenol blue intravenously into cat A. Rous & Gilding [1929] demonstrated that this quantity of dye stains the mouth in less than

a minute, and in 3 min. a maximal staining of the whole animal was reported. In the present experiments within 30 sec. of the dye injection, it appeared in the mucous membranes of the mouth of cat B, and within 3 min. the mucous membranes of nose, mouth and eyes were heavily stained, whereas the injected cat's own head had the merest tinge of the dye after 3 min. This experiment therefore confirms Brodie's opinion that the phenomenon is reflexly produced. However, it is seen from the results of cervical vagal section described above that the vagus cannot be the only sensory nerve involved in the reflex.

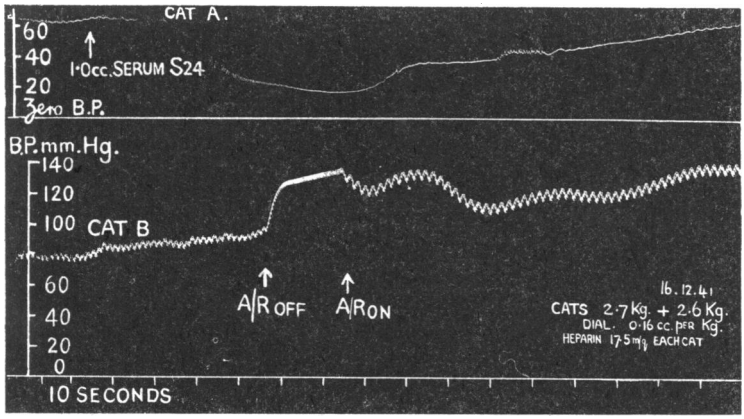

Fig. 11. Crossed circulation of animals' heads. Injection of 1 c.c. of stored human serum into saphenous vein of cat A. (Cat A was breathing normally; cat B was artificially ventilated, and during the crucial experiment the pump ceased working in between the two arrows.) Frequency of heart beat of cat A fell from 40 to 19/10 sec.

Attempts were made to determine the precise location of the vagal nerve endings in the lung concerned in the reflex. Brodie & Russell [1900] showed that inhalation of chlorine, bromine or chloroform vapour, in the dog or cat, could initiate a vagal reflex comparably with the injection of serum. A fine cloud of plasma was sprayed over the tracheal cannula of a cat by means of a 'Mistol' paint sprayer. This had no effect of any kind on the animal, so that the nerve endings in the alveoli of the lung cannot be chemoreceptors sensitive to inhaled plasma. McLaughlin [1933] described an unusually rich distribution of vagal nerve endings in the cat's visceral pleura. It was considered possible that these numerous endings were responsible for the cat's peculiar sensitivity to injections of stored plasma and serum, but the intrapleural injection of stored plasma was without effect.

Leucocytes. The presence of pathological changes in the lung, and the great increase in the number of polymorphonuclear leucocytes in the lung vessels suggested that the clumping of leucocytes might produce minute pulmonary emboli, and thus reflexly cause the cardiac and respiratory changes. Reflex

slowing of the heart, and respiratory effects similar to those produced by plasma injection, have been described by Dunn [1920] and by Binger & Moore [1927], Binger, Boyd & Moore [1927] and Binger, Brow & Branch [1924–5] as a result of multiple minute pulmonary emboli. The emboli were produced in these cases by the injection of starch grains or various plant seeds into the pulmonary circulation.

It was at first thought that plasma influenced the leucocytes in such a way as to make them adhere severally to the walls of capillaries. If, however, the injection was made into the femoral artery, the only difference from intravenous injection noted was a delay in the onset of the symptoms. In order to test the embolism theory still further, recourse was made to the injection of dialysed Indian ink (Higginson's American Drawing Ink, non-waterproof). Rous & Gilding [1929] showed that Indian ink agglutinates *in vitro* and *in vivo* when mixed with plasma. Three c.c./kg. of this ink was injected intravenously and produced no response. Finally, when plasma was injected into the splenic vein, it had no effect on the heart, blood pressure, respiration, etc. but still produced a leucopenia. The missing leucocytes were found when sections of the lungs were examined. This last experiment shows that the sticking of leucocytes within the lung is not responsible for the reflex response to plasma injections; further, that the liver can protect the animal against the toxic substance in stored plasma.

Brodie [1900], quoted by Ponder [1928], demonstrated that one injection of plasma desensitizes the animal for a time (about 40 min.). We have found that this desensitization disappears after an interval of 30–35 min. An injection thereafter will produce all the phenomena described above except the leucopenia, which, however, still persists from the previous injection. The leucocyte count does not return to the pre-injection level until an interval of about 2 hr. has elapsed. Leucocyte counts made 35 min. after plasma are frequently lower than those determined immediately after the injection. This is further proof that the leucopenia is not the cause of the other effects.

Experiments in cross-precipitation with stored human plasma and cats' plasma showed that no precipitins exist in either blood for the proteins of the other. Also, even had precipitins been present in the cat/human plasma, this would not have explained the activity of the stored cat plasma. Cats' red cells were not agglutinated by stored human plasma.

In the above experiments the plasma or serum was injected rapidly into the vein within 2–3 sec. It was decided therefore to try the effect of injections of plasma at a rate more comparable with that of human transfusions. When plasma is run into a vein at a rate of 0·6 c.c./kg./min. for 20 min., the effect on the various systems is much less than that described above, but still there is marked fall of blood pressure and cardiac inhibition at the beginning of the transfusion. The blood pressure rises gradually, but is usually lower at the

end of the injection than before. Dropped beats occur, indicating some injury to the heart. Even though the blood pressure may recover to the initial level, the animals are more sensitive to trauma or haemorrhage in spite of the increased blood bulk.

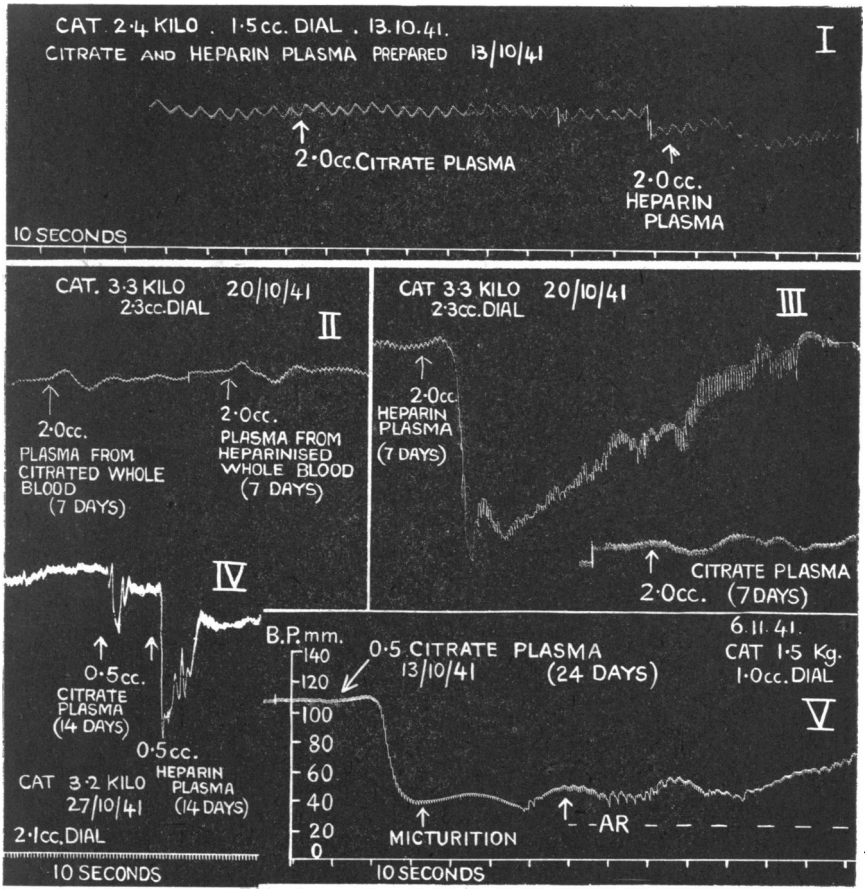

Fig. 12. Time of development of toxicity of citrated human blood and plasma, and heparinized human blood and plasma. I. Effect of 2 c.c. citrated plasma and 2 c.c. heparinized plasma on day of bleeding. II. Effect of 2 c.c. citrated and 2 c.c. heparinized plasma stored in presence of cells for 7 days; cells removed by centrifuge prior to injection. III. Effect of 2 c.c. heparinized plasma and 2 c.c. citrated plasma stored for 7 days. IV. Effect of 0·5 c.c. citrated and 0·5 c.c. heparinized plasma stored for 14 days. V. Effect of 0·5 c.c. citrated plasma stored for 24 days.

Time factor in development of toxicity

Since fresh human plasma is non-toxic to the cat, but becomes so after storage, it was desirable to find out when the toxicity developed. Dr C. R. St Johnston kindly collected blood from donors under the standard conditions

prevailing at the Queen Elizabeth Hospital, citrate being used as the anti-coagulant. Half of the blood was put into the blood bank at the hospital. The other half was centrifuged aseptically at 3000 r.p.m. for 20 min. to remove the corpuscles. A sample of the plasma was then injected into a cat

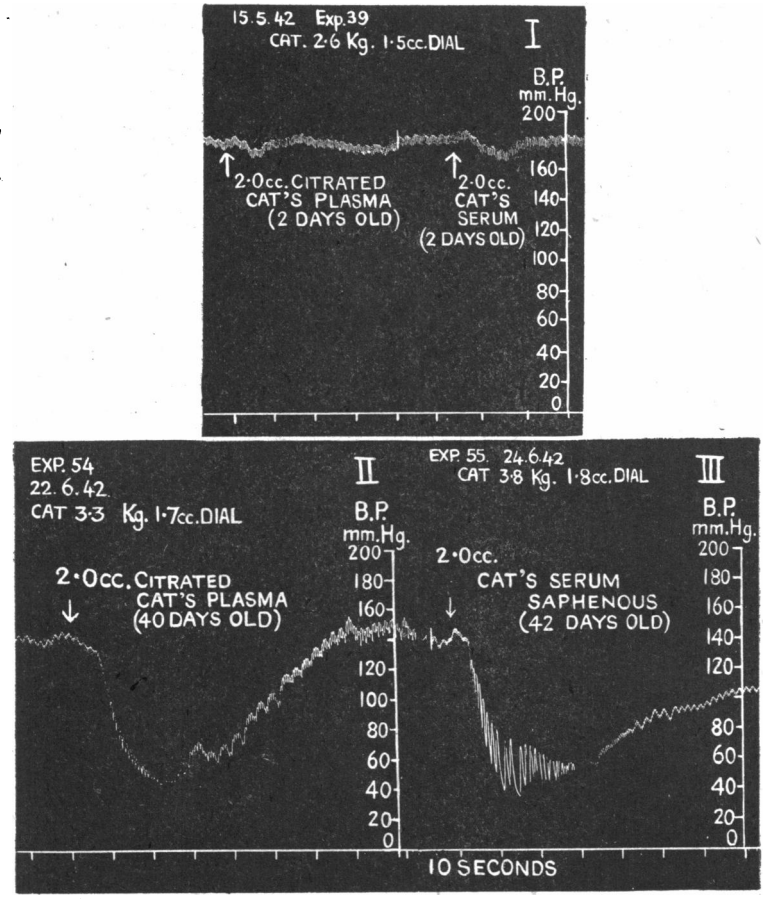

Fig. 13. Development of toxicity in citrated cats' plasma or serum. I. Effect of 2 c.c. cat serum and plasma, 2 days old. II. Effect of 2 c.c. cat plasma, 40 days old. III. Effect of 2 c.c cat serum, 42 days old.

within 45 min. of bleeding. Injections of 0·5, 2 and 10 c.c. of this citrated plasma were completely inactive. The remaining plasma was then decanted aseptically into sterile vessels and stored with the whole blood at the blood bank at the hospital. This plasma and whole blood was thus exposed to the same conditions as that which has been obtained from time to time from stocks at the blood bank. No toxicity had developed in 7 days, a slight toxicity in 14 days, and maximum toxicity in 24 days (see Fig. 12). The

citrated whole blood behaved in the same way as the separated plasma; this differs markedly from the findings with heparinized plasma reported below.

To rule out the effect of the citrate used as an anticoagulant, some blood collected from the same donor at the same time as the above was made incoagulable with sterile heparin (4 mg. in 4 c.c. of saline per 100 c.c. of blood). The subsequent treatment was identical with that for blood collected into citrate.

It will be seen from Fig. 12 (III) that the separated heparin plasma became very toxic within 7 days. We can offer no explanation of this unexpected result. No toxicity developed in the heparinized whole blood until 3 weeks had elapsed, when signs of coagulation appeared.

Subsequently, samples of pooled plasma of different ages (from 7 to 28 days old) were supplied by Dr W. H. P. Cant (Regional Blood Transfusion Officer). The pooled plasma, like that from a single donor, is non-toxic until it is about 21–23 days old.

Stored cats' plasma behaves in a similar way to human plasma, except that it becomes toxic rather more quickly, full toxicity being developed in 2–3 weeks (see Fig. 13).

As stored human serum had been found to be toxic to cats, it was at first thought that the toxicity which develops in stored plasma might in some way be connected with the clotting mechanism, as the heparinized whole blood became toxic at the same time as clotting occurred.

Serum was prepared by recalcification of citrated whole blood and plasma less than 14 days old. This serum was found to be entirely without effect on the animal. Both human and cats' bloods behaved similarly in this respect. Cat's serum, obtained from clotted fresh blood, or by centrifugation of defibrinated blood, develops toxicity at the same time as plasma obtained from the same blood, i.e. within 2–3 weeks.

It is proved therefore that fresh plasma or serum, either human or cat's, is non-toxic to the cat. When the same preparations are tested over a period of one month, a toxicity is found to develop.

Investigation of toxic constituent

Investigations were made with the object of discovering the nature of the toxic constituent of stored plasma and serum.

The test for activity of preparations was the production of a typical response (described above) on their injection into the saphenous vein of the cat. The doses for injection were calculated in terms of the volume of the original plasma from which a given fraction had been prepared.

Histamine extracts. The stored plasma was tested for the presence of histamine by means of extracts made by Barsoum & Gaddum's method [1935].

Fractionation by ammonium sulphate. The plasma proteins were fractionated by the addition of saturated solutions of ammonium sulphate. The ammonium sulphate solution was added to the plasma in the calculated volumes required to produce various percentage saturations; for

full saturation, solid ammonium sulphate was added to the plasma until undissolved crystals remained at the bottom of the vessel.

At each stage of saturation the precipitate was filtered off, sucked as dry as possible on a Buchner funnel, dissolved in water, and dialysed to remove the remaining ammonium sulphate.

Fractionation by lead acetate. Plasma or serum was brought to *p*H 5·0 by the addition of dilute acetic acid, the *p*H being estimated by means of a glass electrode and the Cambridge *p*H meter. Lead acetate in 10% solution was then added in the proportion of 10/100 c.c. of plasma or serum. The *p*H was then raised by stages, by the addition of NaOH to 6·0, 6·5, 7·0 and so on to *p*H 9·0, by which time all the protein had been precipitated. At each stage the precipitate was centrifuged off and taken up in Sorensen's phosphate buffer, *p*H 8·0, the precipitate from 100 c.c. of plasma being taken up in 20 c.c. of buffer. The lead was precipitated as lead phosphate and the protein went into solution. The precipitated lead was removed by centrifugation, and a suitable dose of the protein solution was injected into an animal in order to test its activity. The phosphate buffer itself had previously been found to be non-toxic to the animal.

The lead fractionation was used both on the original plasma and serum and on protein solutions prepared by fractionation with ammonium sulphate.

Fractionation by metaphosphoric acid. In order further to fractionate the proteins of the plasma, or possibly to elute some adsorbed non-protein substance [Rimington, 1941], solutions of 3 *N* metaphosphoric acid were used. The metaphosphoric acid was added to bring down the *p*H of the solution in stages to *p*H 2·0 by which time all the protein in the solution had been precipitated. At each stage the precipitated protein was centrifuged off and taken up in saline, to which a few drops of NaOH had been added to neutralize the acid. The solutions of protein so produced were tested for activity in the usual way.

Results

The extracts of plasma made by Barsoum & Gaddum's method caused only a slight and transient fall of blood pressure in the cat. Further proof that the reaction is not due to the presence of histamine in the plasma, is given by the fact that an injection of histamine gives no protection against an injection of plasma made immediately afterwards. If, however, two injections of equal doses of histamine are made successively into a cat, the second injection produces a much reduced response.

When serum was dialysed overnight against saline, the dialysed serum was active, but the dialysate completely inactive (Fig. 14). Ultrafiltration of stored plasma produced an active ultraconcentrate and an ultrafiltrate which had only a slight depressor activity. Thus it seemed that the toxic substance must be a large molecule or must be adsorbed on to a large molecule.

Plasma was dialysed against a continuous flow of Birmingham tap water (which is almost as pure as distilled water). The precipitated euglobulin was filtered off, taken up in saline, and a portion injected; it proved completely inactive. The dialysed plasma was taken to *p*H 5·3 when a further precipitate of globulin occurred. This, too, proved completely inactive when dissolved in saline and injected. The whole of the activity was still present in the proteins remaining in solution.

The precipitate produced by half-saturating stored plasma with ammonium sulphate was filtered off, taken up in distilled water and dialysed; it was inactive. However, when the filtrate from half-saturation was dialysed and

injected, it was found to have an activity equal to that of the original plasma, when injected in a comparable dose. Attempts were made to fractionate the albumin further by means of ammonium sulphate. It was found possible to bring down all the active substance between 66 and 75% saturation with the salt. The active fraction was further purified by precipitation with lead acetate; the activity went with the proteins precipitating between *p*H 6·5 and 7·0. The protein content of this final active fraction estimated by Kjeldahl's method was found to represent only 8% of the original serum proteins.

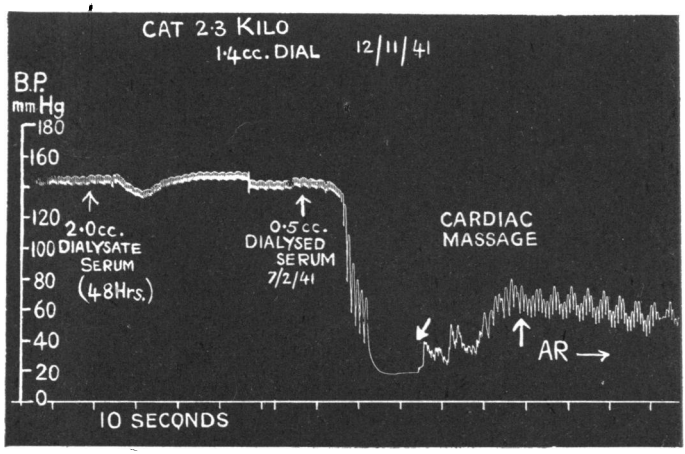

Fig. 14. Activity of dialysed serum and serum dialysate.

The active protein fraction prepared by the lead treatment could not be further purified by treatment with metaphosphoric acid. The active fraction came down between *p*H 3·6 and 2·6. By treatment of the original plasma with phosphoric acid, an active fraction could be separated over the same *p*H range.

It seems therefore that the substance responsible for the toxic action of stored plasma and serum is a protein of the albumin class. This protein is precipitated between 66 *and* 75% *saturation with ammonium sulphate; between p*H 6·0 *and* 7·0 *by the lead acetate treatment; and between p*H 3·6 *and* 2·6 *by treatment with metaphosphoric acid. The active protein represents not more than* 8% *of the total protein of the original serum or plasma. This protein gives a strong Millon's reaction and a feeble glyoxylic reaction.*

Experiments on other animals

Cats proved to be sensitive animals for estimating and analysing the changes that occur when plasma or serum is stored, but it was of interest to see if any of these reactions could be obtained with other animals. For this purpose,

a few experiments were done on dogs, rabbits and human beings with the following results.

Dogs. Doses comparable to those in the cat had little effect, but if 20–50 c.c. of stored plasma was injected intravenously during the course of 1–2 min.

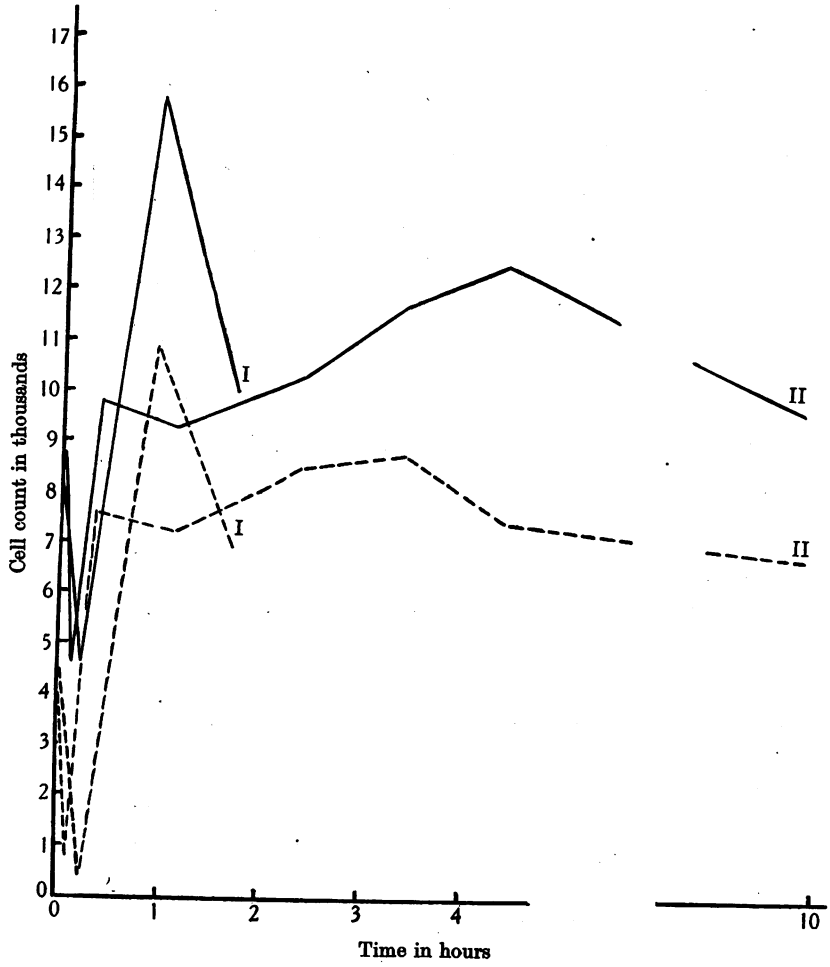

Fig. 15. Continuous lines—total leucocyte count. Dotted lines—polymorphonuclear count. I. Subject—R.A.M.C. II. Subject—H.P.G.

into dogs of 7–15 kg., the blood pressure fell to about 50–70 mm. Hg, returning to normal in 2–3 min. The heart rate was unaffected, but occasional dropped beats occurred. Respiration may cease altogether, or become rapid and shallow for several minutes. There was no micturition, defaecation, opisthotonos or pupillary reaction. There was a slight leucopenia followed by leucocytosis. At autopsy, small congested areas were to be seen in the lungs,

and microscopically the arterioles and capillaries were crowded with polymorphonuclear cells.

Rabbits. Intravenous injections of 2 c.c. stored human plasma had no demonstrable action, except for a momentary slight leucopenia, followed by a pronounced leucocytosis, the cell count returning to pre-injection level in the course of 3–4 hr. The intravenous injection of 20 c.c. of stored plasma in the anaesthetized rabbit had no immediate effect on heart rate or blood pressure; respiration became rapid and shallow; there was no micturition, defaecation, opisthotonos or pupillary reaction. Death occurred after 80 min. The white blood cell count fell from 6750 to 1650 and polymorphs from 4050 down to 82.

Man. Stored plasma known to be toxic to cats was injected into two subjects as follows:

R.A.M.C. (72 *kg.*). 60 c.c. injected into antecubital vein during the course of 20 sec. No effects on respiration or heart rate were observed. The subject said he had 'a muzzy sensation like early anaesthesia, which soon passed off'. He had intense headache next day. The effects on white blood cells are shown in Fig. 15.

H.P.G. (62 *kg.*). 180 c.c. injected into saphenous vein during 2 min. had no effect on the heart rate recorded from the opposite leg, but a few dropped beats occurred; respiration recorded by stethograph showed no effect. During the injection, the subject felt and looked flushed, his lips and tongue felt numb and swollen and his throat dry for 2–3 min. He felt no nausea, but had headache for 24 hr. and general malaise and loss of appetite for 48 hr. The effects on white blood cells are shown in Fig. 15.

Discussion

The above experiments show clearly that a change occurs in plasma or serum when it is stored for upwards of 3 weeks. The nature of this change whether physical or chemical is obscure, but that it is associated with the development of a substance extremely toxic to the cat is proved. The substance appears to be a protein belonging to the albumin class, or else is firmly adsorbed on to such a protein. Brodie [1900] obtained a similar reaction with serum, and stated that this active substance was only produced when blood clots, and also that the interaction of blood corpuscles was a necessary condition for its formation. Neither of these views is tenable in the light of our experiments. The stored plasma and serum used here have been subjected to filtration by Seitz filters which remove all cells and platelets, and yet plasma, without clotting, has been shown to become toxic on storage. Similarly, fresh citrate plasma does not become toxic when clotted by the addition of calcium, a fact which puzzled Brodie. Reid & Bick [1942] suggest that the toxic substance in serum is a protein which they state arises from platelets. This is unlikely

for the following reasons: (1) Plasma which has been passed through a Seitz bacterial filter, and therefore contains no platelets, develops toxicity on storage. There is no difference between the time of development in filtered and unfiltered plasma. (2) Fresh serum, prepared by whatever method, has not been found to be toxic when precautions are taken to remove the fibrin filaments. Stored serum develops activity at the same time as stored plasma. (3) Whole blood contains more platelets than separated plasma. If Reid & Bick's view were correct, the plasma of whole blood should become toxic more rapidly, and should acquire a greater degree of toxicity than separated plasma. This, however, is not the case. Heparinized plasma stored with red cells, i.e. as whole blood, becomes toxic more slowly than separated plasma. There seems to be no difference in the degree of toxicity finally attained in the two cases.

The view that the toxic substance is a protein is supported by the fact that many other proteins and protein derivatives produce effects similar to plasma when injected into cats, and into some other animals. Pollitzer [1886] described a reaction in dogs and cats to the injection of Witte's peptone, and attributed the reaction to the presence in the 'peptone' of certain proteoses. Various other workers, including Chittenden, Mendel & Henderson [1899], Thompson [1896, 1899] and many more recent workers, have shown that the injection of peptones and proteins into cats and dogs produces vasodilatation with a fall in systemic arterial blood pressure. The mode of action of these substances has, however, remained obscure.

A number of authors have described a leucopenia in animals as a result of the intravenous injection of various substances. Bruce [1894] described the disappearance of leucocytes from the peripheral blood of rabbits after the injection of peptone. The polymorphonuclear leucocytes were affected, and their disappearance was not due to destruction, but to their accumulation in the blood vessels of certain organs, chiefly the lungs. There was also an increase in the polymorphonuclear leucocytes in the spleen and liver.

Widal, Abrami & Janovesco [1920] described a leucopenia in dogs, produced by intravenous injection of portal blood obtained during the digestion of proteins. They also noted a leucopenia in dogs in which an Eck fistula was made after a protein meal. They gave the name of 'haemoclastic crisis' to this reaction, which they attributed to the passage of 'albuminoses', products of protein digestion, which are normally removed by the liver, into the general circulation. These authors described a similar phenomenon in liver damage in man, and made this the basis of a test for liver function.

More recently, Staub & Butcher [1938] and Staub, Mezey & Golandas [1938] have described a leucopenia in response to injection of substances other than proteins. Solutions of glycogen, gum arabic, and starch produce this effect in dogs and rabbits. The granulocytes are more affected than the lymphocytes

and the cells which disappear from the blood are found in clumps in the lung vessels. They suggest that this is a result of the agglutination of leucocytes which have phagocytosed the injected macromolecules, and thus lost the power of passing through the lung capillaries. Vejlens [1938] noted a leucopenia as a result of the intravenous injection of various hydrophil colloids into guinea-pigs, rabbits and cats, and also as a result of decreased velocity of blood flow in these animals. He attributed the leucopenia to changes in the hydraulics of the circulation which caused the white blood cells to become 'marginal flowing' instead of flowing in the axial stream, and also made them adhere to the vessel walls. These changes in the distribution of white cells were considered by him to be part of the body's defence reaction to the injection of foreign substances.

In view of the similarities between the grosser symptoms of plasma injection and of anaphylactic shock, it is of interest to note that similar changes occur in the distribution of the blood cells in the two cases.

Biedl & Kraus [1909] described the occurrence of leucopenia in anaphylactic shock, and Andrewes [1910] noted this both in anaphylaxis in rabbits and as a result of injection of various bacteria intravenously in these animals. He noted, too, that polymorphs were present in excessive amounts in the liver.

In anaphylactic shock in dogs, Dean & Webb [1924] noted a polymorphonuclear leucopenia followed by leucocytosis. Webb [1924] subsequently showed that the leucopenia was due to polymorphonuclear cells adhering to vessels within the lung. Deep cuts in the lung substance, which produced marked haemorrhage, failed to release the adherent leucocytes. Dean [1922], in a case of anaphylaxis in man, reported that the sinusoids of the liver and vessels of the lungs were packed with white cells. Amongst the leucocytes were a large number of eosinophils. In addition he noted rupture of the alveolar walls in certain areas, but, as artificial respiration had been performed, this might possibly be ascribed to external trauma.

The injection of atropine very much decreases the cat's response to stored plasma. Auer [1910] showed that atropine diminishes anaphylactic shock in the guinea-pig. Lumière & Meyer [1938] showed that this was also true for the anaphylactoid response to injection of ground pumice stone. It seems possible, therefore, that anaphylactic shock, as well as the anaphylactoid responses to injections of stored plasma and serum, and of particulate matter, may in part consist of a reflexly evoked outburst of parasympathetic activity which is paralysed by atropine.

The results of the experiments indicate that the response to injection of stored plasma or serum is a reflex one. The reflex is stimulated by some constituent of the injected fluid and the efferent path is largely, but not entirely, via the parasympathetic nervous system. The muscular movements in the anaesthetized and decerebrate animals show that other parts of the nervous

system are involved. The receptors are mainly in the lungs, but, as the pupillary reactions to stored plasma in vagotomized animals show, part of the sensory pathway is from the abdominal area via the splanchnic nerves, possibly the pathway demonstrated by Bain *et al.* [1935] which they showed carried pupillo-dilator impulses from the intestines.

The speed with which the respiratory rhythm changes to that of the Cheyne-Stokes type is too quick to be produced by any oxygen lack or accumulation of CO_2. The records of intrapleural and pulmonary arterial pressures (Figs. 2, 5) suggest that the volume and pressure of blood in the lungs increases after the injection of stored plasma; whether this increased blood pressure and volume or the plasma itself stimulates the vagal lung receptors is uncertain. Partridge [1939] believes that the efferent vagal fibres which cause reflex acceleration of respiration in the dog are stimulated by circulatory changes in the lung. Hammouda, Samaan & Wilson [1943], however, deny this. Daly, Ludány, Todd & Verney [1937] showed that a rise in pulmonary arterial pressure may cause variations in respiratory rate; vagotomy abolishes this response. No effect is produced by making the animal breathe a fine cloud of plasma or by injecting plasma into the pleural cavity. If, therefore, the nerve endings in the lung are sensitive to a chemical substance, these endings must be in close apposition to the lung vascular system.

The 'immunity' to further injections of stored plasma mentioned above, which lasts for a period of about 35 min., is difficult to explain on any immunological basis. But, as the response is reflex in nature, the 'immunity' is doubtless due to a fatigue of sensory endings. This view is borne out by the effects of operative interference in the chest and overventilation by artificial respiration noted by Brodie [1900], and also by the fact, noted above, that the effects on respiration last for a period up to 20 min.

It is probable that the acute symptoms of massive pulmonary embolism in man are a manifestation of this reflex.

Summary

1. Plasma or serum develops a toxicity on storing for 3–4 weeks. Stored human or cat plasma may kill a cat when so little as 0·5 c.c. is injected intravenously.

2. Clotting of plasma plays no part in the development of toxicity. Breakdown of platelets is not responsible, since filtered plasma becomes toxic at the same time as unfiltered plasma.

3. The activity is associated with a protein of the albumin class.

4. The effects of intravenous injection in the cat are: vagal inhibition of the heart, fall of blood pressure, altered respiration, increased peristalsis, micturition, defaecation, vomiting, constriction of the pupil, opisthotonos,

all of reflex origin, and a leucopenia which is followed, after some hours, by a leucocytosis.

5. The main sensory nerves for this massive reflex are the vagal lung fibres, and most of the effects are abolished by vagotomy or by atropine.

6. The mode of stimulation of the sensory nerve endings has not been determined, though it is possible that pressure changes in the pulmonary circulation may in some way act as a stimulus.

7. The changes in white blood cells, as well as the more gross responses to the injections, are very similar to those occurring in anaphylactic shock and in anaphylactoid conditions. These different conditions may merely represent different methods of eliciting a general physiological response to the injection of foreign matter into the circulation.

The authors wish to thank Dr R. A. M. Case for kindly acting as a subject; Dr W. H. P. Cant for providing the samples of human plasma and serum and for his continued interest in the work; Dr C. R. St Johnston for the collection of special samples of blood; Dr F. Jacoby for filtering samples of plasma from time to time; and Mr K. A. Webb for his invaluable technical assistance.

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