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THE UPTAKE AND TRANSMISSION OF PROTEIN BY
THE GUT OF THE NEONATAL RAT

by

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Thesis submitted to the University of Nottingham
in application for the degree of Doctor of
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CONTENTS

	Page
CHAPTER ONE INTRODUCTION	1
CHAPTER TWO MATERIALS AND METHODS	19
a) Animals	19
b) Preparation of labelled proteins and polyvinylpyrrolidone	19
c) Counting equipment	20
d) Operative procedure	20
e) Determination of the distribution of radioactivity in the body	22
f) Ultracentrifugation	23
CHAPTER THREE DETERMINATION OF PLASMA VOLUME	25
Introduction	25
Methods	25
Results	26
Discussion	29
CHAPTER FOUR THE UPTAKE AND TRANSMISSION OF PROTEINS BY NEONATAL RAT ENTEROCYTES	32
Introduction	32
Methods	32
Results	35
Discussion	45
CHAPTER FIVE DEGRADATION OF IgG IN THE SMALL INTESTINE	50
Introduction	50
Methods	50
Results	54
Discussion	58
CHAPTER SIX KINETIC AND SATURATION STUDIES ON THE TRANSMISSION OF RAT IgG ACROSS THE PROXIMAL SMALL INTESTINE	63
Introduction	63

	Page
Methods	63
Results	64
Discussion	72
CHAPTER SEVEN SUBCELLULAR FRACTIONATION STUDIES	77
Introduction	77
Methods	78
Results	86
Discussion	91
CHAPTER EIGHT DISCUSSION	94
REFERENCES	109

CHAPTER 1

INTRODUCTION

At birth, the capacity of newborn mammals to actively produce antibodies in response to an antigenic stimulus varies from species to species. Some mammals with long gestation periods are immuno-competant at birth, however the majority have only a rudimentary immune system.

Neonates lacking active immunity have little resistance to infection during this first critical period of life. Immuno-competant neonates are also vulnerable. Before birth they are largely prevented from coming into contact with antigens, so although they have an active immune system, they are generally not primed or sensitized to react rapidly to an antigenic stimulus.

In both cases antibodies actively produced by the mother are transmitted from her circulation to that of her offspring, and so provide them with a ready made immunity to those antigenic stimuli to which she has been exposed.

This materno-foetal transmission of antibodies can occur either before or after birth, or at both these times. Human infants and the young of monkeys, rabbits and guineapigs receive their full complement of maternal antibodies before birth. This process involves the passage of these antibodies across the chorio-allantoic placenta in primates and the yolk sac splanchnopleur in the rabbit and the guineapig.

In contrast to the previous group, the young of cattle, sheep, goats, horses, donkeys and pigs are born with no passively acquired immunity. In these animals the mother's milk is rich in antibodies, and during the first few hours of suckling these antibodies are transported to the circulation of the neonate by the action of the digestive tract. This ability of the neonate's gut to transmit intact immunoglobulins is transitory and lasts from 24-36 hours after birth depending upon the

species.

The young of rats, mice, hedgehogs, cats and dogs are capable of receiving maternal antibodies both before and after birth. Although a significant amount is transmitted to the foetus before birth, the majority is transmitted after birth by way of the milk. In rats, mice and hedgehogs, this postnatal transmission of antibodies may continue for up to 21 days, 16 days and 40 days respectively.

Investigations into this vital phenomenon began in the late nineteenth century. In 1880 Chauveau found that by vaccinating the slightly susceptible Algerian sheep with Anthrax organisms during gestation, their offspring developed an immunity to this organism. These findings were later confirmed by the work of Arloing, Cornevin and Thomas (1882) and Kitasato (1889).

The publication, in 1892, of the results of Ehrlich's basic studies marked a new epoch in the development of the subject. He carried out immunization experiments by feeding mice the toxalbumins abrin, ricin and robin, and found that the young of immune mothers acquired an immunity to these toxins. He considered this immunity to result from the passage of specific antibodies to the foetus through the placenta, and *post-partum* to the neonate via the milk. He demonstrated that the young of non-immune mothers, when suckled on immune mothers also acquired immunity. He further showed that highly immune males when crossed with normal females did not produce immune young. Ehrlich clearly stated that the immunity was transmitted to the young physiologically and was not inherited genetically. This last point caused considerable controversy, and in 1894 Ehrlich repeated his work to confirm his findings. In subsequent years they were confirmed by many workers including Wernicke (1895), Vaillard (1896), Remlinger (1899), Dieudonné (1899) and Bulloch (1902).

During the next thirty years there was considerable activity in this

area of research, mostly centred around the problem of the permeability of the placenta to antibodies. One finds in the contemporary literature placental transmission being both positively affirmed and vigorously denied.

The researches into the transmission of passive immunity to *Trypanosoma lewisi* in rats by Minning (1936) and Culbertson (1938, 1939a, 1939b, 1940) laid the foundations for subsequent investigations in this field. In his preliminary studies, Culbertson (1938) showed that young rats born of mothers which had recently recovered from an infection of *T. lewisi* were themselves immune to this pathogen for the first few weeks of life. Young born of non-immune mothers soon developed immunity when fostered upon immune mothers. He concluded from these experiments that protection from the pathogen was being transmitted to the neonate by antibodies transferred from the maternal circulation by way of the placenta before birth and via the milk after birth. These antibodies were specific for *T. lewisi*, as he demonstrated that the young of mothers immune to *T. lewisi* although resistant to this pathogen were susceptible to *T. cruzi*. This ability to absorb antibodies from the mother's milk appeared to cease at about 21 days *post partum*.

Culbertson (1939a) showed that a mother passively immunized by an intra-peritoneal injection of serum from a rat that was actively immunized to *T. lewisi*, could transfer this immunity to her young. He concluded from these findings that the antibody which was being transported to the neonate was identical to that found in the circulation of the parent. He also demonstrated that suckling rats could be immunized by an orally administered dose of immune serum (1939b).

In the years following Culbertson's papers his findings were confirmed many times. Kolodny (1939) repeated the experiments using *T. cruzi* as the antigen, and found that the degree of immunity acquired

by young rats was inversely related to the size of the litter. The post-natal transmission of passive immunity to the following antigens was subsequently demonstrated; *Recketttsiae* (Worth 1951; Jo 1953), *Plasmodium bergi* (Bruce-Chwatt and Gibson 1956; Terry 1956), *Salmonella* (Halliday 1955b), *Toxoplasma* (Lewis and Markell 1958) and *Bruceella abortis* (Halliday and Kekwick 1960).

Significant advances in our understanding of the mechanisms of transmission came from the work of Halliday (1955a, b). Using a stomach tube he fed precise doses of immune serum to suckling rats, and examined the transport of antibodies to the circulation from the gut. He found the transport mechanism to be very rapid, and was able to detect antibodies in the serum only 30 minutes after feeding. The serum titre increased thereafter, reaching a maximum value within three hours, and remaining at that level for two days.

Site of transmission

Further insights into the mechanisms of gamma globulin transmission came from the electron microscopical studies of Clark (1959), who examined the ingestion of proteins and colloidal materials by the cells of the small intestine. Using a fluorescent antibody technique he was able to demonstrate in suckling rats and mice the pinocytotic absorption of bovine gamma globulin by the cells of the jejunum and ileum, but not the duodenum.

Initially attention was focused upon the ileal enterocytes as the site of gamma globulin uptake. These cells exhibit a striking morphology as noted by many workers (Graney 1968; Kraehenbuhl, Gloor and Blanc 1966, 1967; Kraehenbuhl and Campiche 1969; Rodewald 1973). The ileal cells possess a large supranuclear vacuole and an intercommunicating system of apical canaliculi. These tubules have a distinct membrane structure

composed of small regularly arranged particles, 7nm in diameter, on the luminal surface of the membrane. The ability of this system of apical tubules to endocytose proteins present in the intestinal lumen was originally considered to represent the pathway for gamma globulin transport (Clark 1959; Kraehenbuhl, Gloor and Blanc 1967; Kraehenbuhl and Campiche 1969; Graney 1968). More recently however, it has been demonstrated that these ileal enterocytes do not play a part in the selective transmission of gamma globulins. They have a strictly non-selective phagocytic function (Hugon 1971; Rodewald 1973; Worthington and Graney 1973). The transport of all tracers to the supranuclear vacuole and the presence of lysosomal enzymes within the vacuoles (Cornell and Padykula 1969) support the biochemical evidence that almost all the protein entering these cells is digested (Morris and Morris 1977a, 1977b, 1978).

It has now been quite firmly established that the site of selective gamma globulin transmission in the rat is restricted to the duodenum and proximal jejunum (Rodewald 1970 ; Mackenzie 1972; Morris and Morris 1974, 1976; Morris, B. 1975; Jones 1976b; Waldmann and Jones 1976). There is some evidence however that the ileum may also be active in this role (Hemmings and Williams 1977), although this hypothesis does not enjoy wide support. The cells of the proximal region of the small intestine are functionally and ultra structurally similar to other IgG transporting tissues such as the rabbit yolk sac endoderm. These columnar absorptive cells have a well developed brush border, and throughout the apical cytoplasm have been shown to contain tubular and coated vesicles (Rodewald 1970, 1973; Moxon *et al* 1976). Tubules and vesicles have a cytoplasmic membrane coat, which in the case of the former is only evident during their formation at the microvillar surface.

Selectivity

Antibodies produced in species other than the rat can be transmitted from the gut to the circulation of suckling rats. Bessis and Freixa (1947a, 1947b) showed that haemolytic disease was induced in rats under 22 days of age by the oral administration of rabbit anti-rat red cell serum. It was quite evident that the resulting haemolysis was a consequence of the transport of immunologically intact rabbit immunoglobulins across the rat intestine.

Halliday (1955a) pursued this line of inquiry by examining the intestinal transmission of antibodies from sera prepared in several different species. Anti-*Salmonella pullorum* antibodies produced in mice were transmitted almost as readily as those produced in rat and as readily as rat incomplete agglutinins to sheep red cells. Anti-*Bruceella abortus* antibodies produced in rabbit were also transmitted, but only at a relative concentration (C.Q.) of $\frac{1}{8}$ that of rat antibodies. However, anti-*B. abortus* antibodies produced in cows and fowl were not transmitted in detectable quantities.

Hemmings and Morris (1959) confirmed this phenomenon of selectivity in mice by showing that antibodies from guinea pigs hyperimmune to *S. pullorum* were transmitted about half as readily as those from hyperimmune mouse anti-*S. pullorum* serum, and those from hyperimmune rabbit anti-sheep red cell serum were transmitted about a quarter as readily.

Subsequent studies by Halliday and Kekwick (1960) showed that the amount of antibody transmitted to the circulation by the gut of the young rat depended upon whether the antibody had been produced in the primary immune response or after hyperimmunization, and also upon the nature of the antigen used. Mothers that had been actively immunized against *S. pullorum*, transmitted to their young a smaller proportion of antibody during the primary immune response than when they were hyperimmune.

This was not due to differences in the antibody titre of the mother's milk, but to a reduced capacity of the neonate's gut to absorb the antibodies. This was demonstrated by oral administration of immune serum taken after primary and hyperimmunization. The former attained a much lower relative concentration in the young rat's serum even when the hyperimmune serum was diluted with non-immune serum to a titre corresponding to that of the primary immune serum. Halliday showed also that antibodies produced from the serum of rats hyperimmunized to *B. abortis* were not transmitted to the circulation as readily as those from hyperimmune *S. pullorum* serum.

The explanation for these differences was that the antibodies present in the primary immune response to *S. pullorum* and in hyperimmune anti-*B. abortis* serum were mainly of the beta-globulin class. Whereas those produced in the hyperimmune response to *S. pullorum* were of the gamma-globulin class (IgG). The gut of the young rat selectively transported antibodies of the IgG class rather than those of the IgM class.

Having established that the process of selection involved the gamma globulin component of the serum, it was of interest to discover which part of the IgG molecule was responsible for this phenomenon. It was about this time that Porter (1958, 1959) succeeded in splitting rabbit IgG molecules into Fab and Fc fragments using papain. These fragments were trace-labelled with ^{131}I and their rates of transmission from the rabbit uterine cavity to the foetal circulation were compared with that of whole rabbit IgG similarly labelled (Brambell, Hemmings, Oakley and Porter 1960). It was found that the Fc piece was transmitted almost as readily as the whole molecule, whereas the Fab fragments were transmitted a tenth as readily.

Interference

The phenomenon of interference was demonstrated by the work of I. G. Morris (1956, 1957) on the induction of haemolytic disease in young mice by the oral administration of rabbit-anti-mouse red cell serum. He found that dilution of the minimum lethal dose with saline did not reduce the effect of the antiserum. However, mixture of the antiserum with non-immune serum produced a dilution effect. The lethal dose of a 1:1 mixture of immune and non-immune sera was double the volume of the lethal dose of immune serum. The conclusion reached from these findings was that the non-immune serum was competing with the antiserum for uptake into the circulation.

A similar phenomenon was shown to occur in rats by Halliday (1958). He extended the study to demonstrate that the degree of competition between the antiserum and the non-immune serum depended upon the species of origin of the latter.

Brambell, Halliday and Morris (1958) subsequently went on to investigate which component of the serum was responsible for interference. Examination of the capacity of various serum fractions to cause interference revealed that it was the IgG fraction which was active in this respect.

In further studies, Morris (1963) examined the effects of Fab and Fc fragments produced by papain digestion, on the transmission of guinea-pig antibodies to young mice. He found no detectable interference by the Fab piece, whereas the Fc piece was 3.5 times more effective than the intact molecule. Thus the capacity to interfere with the transmission of another gamma-globulin resides in the same part of the molecule that is involved in selective transmission.

Saturability

Halliday (1958) examined the relationship between the dose of immune serum administered and the concentration of antibody subsequently attained in the circulation. The relative concentration of antibody in the serum appeared to be linearly and directly related to the size of the dose up to a certain maximum value. Larger doses could not produce an increase in concentration. At doses below the maximum, 33% of the administered gamma globulin was transmitted intact to the circulation, and this value declined for doses greater than the maximum. Halliday concluded from these studies that the IgG uptake mechanism was saturable.

These findings on selectivity, interference and saturability have been confirmed many times (Halliday 1955a, 1958; I. G. Morris 1964, 1965, 1967, 1969, 1976; Jones and Waldmann 1972; Guyer, Koshland and Knopf 1976).

Receptors

A considerable part of the important work performed between 1950 and 1960 was under the supervision of F. W. Rogers Brambell. Towards the end of this period it became clear to Brambell and his colleagues that the phenomena of selective gamma globulin transmission and interference must involve some form of molecular recognition. These considerations led Brambell's group to the concept of a gamma globulin receptor which could account for both selectivity and interference. Receptor mediated transport also explained the saturability of the IgG uptake mechanism which had been observed by Halliday (1958).

In 1958, Brambell first formulated his receptor hypothesis. It stated that immunoglobulins were taken up in a non-selective manner, by the endoderm cells of the yolk-sac of the foetal rabbit at least (Hemmings 1957), and that such uptake is probable in other species also.

The mechanism of uptake was considered to be pinocytotic. To account for the phenomena of selectivity and interference it was assumed that the absorptive cells of the rabbit yolk-sac and the intestine of the suckling rodent possessed receptors adapted to fit homologous gamma-globulins. These receptors were also capable of binding to heterologous gamma-globulins. The resemblance in surface configuration between gamma-globulin molecules from different species could then be the basis of selection, if the probability and duration of attachment of the gamma-globulins to the receptors were related to the goodness of fit. This hypothesis accounted for interference as competition between gamma-globulins for the receptor.

At this stage in the development of the receptor hypothesis, there was little indication of the location of the receptors within or on the absorptive cells, nor of their nature. Most of the experimental work leading up to the first Brambell Hypothesis made use of immunological methods of labelling serum proteins. A whole range of immunological and serological techniques such as titration, skin and anapylactic tests, immunodiffusion, immunoelectrophoresis and the use of fluorescent antibodies, were adapted to the recognition of antibody/antigen reactions. However, major advances in our understanding of the gamma-globulin receptor resulted from the use of radioactively labelled molecules. These radio-labelling techniques proved more sensitive than the immunological methods of detection.

Using ultracentrifugation techniques Jones and Waldman (1972) showed that after uptake of IgG into the proximal enterocytes, the antibody molecule formed complexes of a molecular weight greater than that of the original antibody molecule. They considered this to represent an IgG-receptor complex. Careful kinetic studies (Morris, I. G. 1964, 1976; Guyer *et al* 1976) showed clearly that there appeared to be a single class of

specific receptors which recognised Fc determinants.

More recently (Rodewald, 1976a, 1976b, 1980a) morphological and biochemical binding studies have confirmed the presence of the IgG Fc-receptor in proximal enterocytes. This receptor has been shown to bind selectively to the Fc portion of all IgG subclasses, but binds to neither IgA nor IgM (Borthistle et al 1977). The receptor is said to be sensitive to digestion by trypsin, and it disappears three weeks after birth - at the time when this region of the gut ceases to transmit intact IgG.

The work of Jones and Waldmann (1972) demonstrated that IgG-membrane complexes were present in gut homogenates at pH values below 6.5, but not at pH 7.4 or higher. This implied that IgG could bind to the receptor on the luminal cell surface which had a pH of about 6.0 (Rodewald 1976c) and could be released on the abluminal cell surface on exposure to the extracellular plasma which was considered to have a pH of 7.4.

Using IgG-peroxidase conjugates, Rodewald (1976a, b, 1980a) showed convincing morphological evidence for the selective binding of IgG at the apical surface of proximal enterocytes at pH 6.0. These observations have been confirmed by Nagura, Nakane and Brown (1978).

On examination of the binding of IgG-horseradish peroxidase conjugates to isolated cells, Rodewald (1976a, 1980a) found IgG receptors over the entire surface of the plasma membrane which shared the same pH sensitivity to binding. These receptors did not appear specifically associated with coated vesicles attached to the surface. Rodewald suggested that these receptors and IgG-receptor complexes were transported across the cell within several distinct membrane compartments, from the apical membrane to the tubular and coated vesicles, and then to the abluminal membrane. A shuttling of IgG receptors in both directions was proposed which because of the effect of pH on binding would result in a net transport of IgG in an abluminal direction (Rodewald 1980a).

Proteolysis

A major influence on the development of the receptor hypothesis arose from the realization that the transmission of antibodies across the rabbit yolk-sac and the intestine of the suckling rodent was accompanied by considerable degradation of antibody (Hemmings 1957; Bangham and Terry 1957).

Brambell, Halliday and Hemmings (1961) examined this situation by feeding ^{131}I labelled bovine gamma-globulin to 14 day old rats. They showed that four hours after feeding, only 10% of the dose was recoverable as intact IgG in the circulation, even though 70% of the dose had been absorbed from the intestine. Thus about 86% of the absorbed dose had been degraded.

The method of digestion in the neonatal gut appeared to differ from that in the adult gut, where most digestion is intraluminal. As shown by Jones (1972, 1978) the luminal contents of the neonatal gut exhibit only low levels of proteolytic activity within the physiological pH range of the gut contents (Rodewald 1976c). However, there is considerable proteolytic activity in the intestinal cells, particularly in the ileal region (Noack 1966; Cornell and Padykula 1969; Williams and Beck 1969; Bainter and Juhász 1971; Jones 1972, 1978). The possible implication of these studies is that as milk passes down the gut, IgG is removed by the duodenum and jejunum for transmission intact to the circulation, and the remaining material is later absorbed and digested in the ileum (Rodewald 1973).

Quantitative studies conducted by Morris and Morris (1976, 1977a, 1977b, 1978) compared the transport and digestive capabilities of both proximal and distal regions of the gut. The proximal enterocytes appeared to transport intact to the serum up to 40% of the IgG removed from the intestinal lumen, the remainder being digested. Distal enterocytes on the other hand degraded over 98% of the absorbed IgG.

Evidently the proximal region of the gut is the site of transmission of intact IgG to the circulation, however a considerable amount of IgG breakdown also occurs. The proportion of the IgG that is broken down appeared to depend on the concentration of the dose administered. Hemmings (1975a) showed that as the concentration of the dose was increased, so the proportion of the dose that was degraded also increased. This suggested that the capacity of the cell to transport intact protein was saturable and that excess protein was digested.

The colostrum of pigs and cats (Laskowski, Kassell and Hagerty 1957; Bainter 1973), cows (Laskowski 1951) and lambs (Bainter 1976) has been shown to be rich in trypsin inhibitor. It has been claimed that the function of these trypsin inhibitors is to prevent the digestion of proteins destined for transmission to the circulation, (Bainter 1973; Laskowski 1958; Westrom 1979; Nordbring and Olsson 1958a, 1958b; Carlsson *et al* 1975). However, Jones (1980) was unable to stimulate the transmission of IgG across the suckling rat gut by the addition of trypsin inhibitor. It appears that trypsin inhibitor may only be effective in increasing intestinal uptake of protein in those mammals where uptake is non-selective. In selective uptake, the IgG is presumably protected from digestion by binding to receptors.

The termination of transmission

The termination of the capacity to transmit immune globulin from the gut to the circulation occurs between 18-21 days of age in the rat (Halliday 1955a). The mechanisms responsible for terminating the permeability of the neonatal gut to IgG have been the subject of much speculation.

Halliday (1956) demonstrated that 21-25 days after birth, the immune mother was still actively secreting antibodies into her milk, but by this

time the neonate was no longer capable of transmitting intact antibodies to its circulation. Young rats aged 21 days or more were unable to absorb antibodies from mothers who had been lactating for less than 18 days, and were also unable to absorb antibodies from their own immune mothers. Conversely, 17 day old rats, made to suckle immune mothers who had been lactating for 16-23 days, were still able to take up antibodies from the milk of these mothers. Halliday concluded from these findings that there was nothing in the mother's milk, between 16-23 days *post-partum*, which could cause closure.

The possibility that the taking of solid food might be the stimulus for closure was examined by Halliday (1956). He demonstrated that feeding 12 day old rats on a solid food diet did not impare their ability to transmit antibodies from the gut to the serum.

Studies on the levels of alkaline phosphatase in the neonatal mouse duodenum (Moog 1951) showed that there was a large increase in the amount of this enzyme between the age of 13-18 days. It was subsequently observed that this increase could be induced prematurely by the administration of steroid hormones (Moog 1953; Moog and Thomas 1955). The coincidence in time of the marked rise in alkaline phosphatase levels and the closure of the gut to the transmission of intact IgG, led workers to suggest that there might be a causal relationship.

Halliday (1959) repeated the work of Moog on the young rat, and found an equivalent increase in alkaline phosphatase between 18-23 days of age. Administration of very large doses of deoxycorticosterone acetate or of cortisone acetate to young rats brought about a premature rise in alkaline phosphatase and precocious termination of antibody transmission. Administration of aldosterone, progesterone, testosterone and stilboestrol had no such effect. However premature weaning of the young had the same effect as treatment with deoxycorticosterone acetate. Halliday suggested

that the stress of weaning may result in adrenal secretion and hence the termination of transmission. The fact that transmission is terminated earlier in runts (Kyffin 1967) is open to a similar interpretation.

The observation that the injection of large amounts of glucocorticoids could cause premature closure (Clark, 1959; Halliday, 1959; Daniels *et al* 1973a), together with the finding that plasma corticosterone levels increased at the time of closure (Daniels *et al* 1972), suggested that closure might depend upon the secretions of the adrenal cortex. However subsequent results have shown that such a dependence is not absolute. The phenomenon of closure could not be prevented by adrenalectomy, but only delayed for 2-4 days (Daniels *et al* 1973b; Morris and Morris 1981a).

Morris and Morris (1980) stressed 12-16 day old rats by daily removing them from their mother for 18 hour periods. This treatment led to precocious closure, however the mean plasma corticosterone levels in such animals were not significantly different from those of normal young rats. Thus in the context of the termination of antibody transmission, the evidence for the involvement of an adreno-cortical trigger is conflicting. Since, in all of the relevant studies unphysiological doses of glucocorticoids have been used to produce measurable results - these effects are more likely to have been produced by unspecified pharmacological actions of the preparations, rather than to the mimicking of the role of the adrenal cortex in normal development.

It has been demonstrated that administration of thyroxine to the suckling rats caused a precocious increase in the plasma corticosterone levels (Malinowska 1974), and a premature cessation of macromolecular uptake by the intestine (Malinowska, 1974; Chan, 1973; Daniels *et al*, 1973a). Closure may be the result of some form of mutual interplay between the thyroid and the adrenals, which is not yet fully understood.

Models for the mechanism of transmission

In the light of advances made in the field, the original "Brambell Hypothesis" (1958) was restated many times (Brambell, 1963; Brambell, Hemmings and Morris, 1964; Brambell, 1966) and by 1970 a final evaluation of the hypothesis was made (Brambell 1970). The originator of the hypothesis hoped it would account for the phenomena associated with uptake, but he also realized that it left many questions unanswered and would inevitably have to be modified. The observed facts that had to be accounted for by the hypothesis included;

- a) selection, operating not only between very different molecules such as IgG and albumin, but also between different IgG molecules,
- b) interference by one IgG molecule with the transmission of another IgG molecule,
- c) both selection and interference being associated with the Fc terminal of the IgG molecule,
- d) a substantial proportion of the IgG taken up by the enterocytes being digested,
- e) absorption by pinocytosis being the only demonstrated means of uptake by the cells.

The working hypothesis proposed by Brambell stated that receptors were present on the surface of the microvilli, and are carried into the cell by invagination of the caveoli. These pinocytotic vesicles subsequently fuse with lysosomes, and material which had been fortuitously interiorized by pinocytosis but was not receptor-bound was subjected to proteolysis in the newly formed phagolysosome. The receptor-bound protein was supposedly protected from digestion, and was eventually discharged intact from the cells by exocytosis into the intercellular space.

This model left many questions unanswered, and its validity has since been challenged many times. Wild (1976) working on rabbit yolk-sac

endoderm, demonstrated using fluorescent labelled anti-cathepsin antibodies, that lysosomes could not be detected fusing with and exocytosing at the lateral or basement membrane of the cell. Secondly no totally satisfactory explanation has been advanced for the way in which binding of IgG to a receptor protects it from proteolytic attack. Finally there seems to be no reason why selection should be achieved at the phagolysosomal level, when it has already been achieved at the receptor binding level (Rees and Wallace 1980).

Wild (1975) proposed that selection occurs at the level of receptor binding at the cell surface, and that no significant quantity of non-receptor bound protein is included in the pinocytotic vesicle. He claimed that the vesicles involved in receptor mediated protein uptake are in fact coated, and their cytoplasmic coat acts as a fender to prevent fusion with lysosomes. If this is the case then a mechanism still has to be devised for allowing the coated vesicle to fuse with the basal or lateral cell membrane but at the same time preventing fusion with lysosomes.

Rodewald (1973) suggested that highly specific IgG receptors are present on the apical cell surface and within endocytic tubular vesicles, which at the time of formation appear to have a cytoplasmic coat. The IgG molecules are transferred from the tubular vesicles to spherical coated vesicles, which with the aid of a pH shift, discharge the IgG at the lateral plasmalemma by reverse pinocytosis. Rodewald claimed to be unable to detect the fusion of coated vesicles with lysosomes.

A totally different explanation has been suggested by Hemmings (1975a, 1976). The selective transport of IgG was not considered to involve a surface receptor, and the involvement of coated vesicles was thought to be serendipitous. Uptake was said to be non-specific; all protein binding to the sticky glycocalyx with equal facility and subse-

quently being pinocytosed. Once inside the cell, the pinocytotic vesicle erupts, so releasing the protein into the cytoplasm. Selection then operates at the basolateral membrane by means of a diffusion carrier system, which must incorporate some form of molecular recognition ability.

Aims of the thesis

The studies reported in this thesis deal with the *in vivo* transmission of homologous and heterologous IgG and other proteins across the proximal small intestine of the suckling rat.

The uptake of these labelled macromolecules from the intestine was monitored, and the subsequent appearance of radioactive material in the serum, viscera and carcass was examined by ultrafiltration and ultracentrifugation techniques.

The rate of transmission of intact IgG and IgG breakdown products across the intestinal barrier was measured with the intention of obtaining further information about the mechanisms of immunoglobulin transport.

The significance of the digestive activity of the suckling rat gut to the process of antibody transmission was also assessed. This was done by gel filtration analysis of the radioactive material present in the gut wall and wash after the intraluminal injection of labelled rat IgG. The ability of trypsin inhibitor to stimulate the transmission of intact IgG was investigated, and the possibility that the IgG receptor on the surface of proximal enterocytes was trypsin sensitive was also examined.

Attempts were made, using a variety of subcellular fractionation techniques, to isolate and identify the organelles involved in the receptor mediated transcellular transport of IgG.

CHAPTER 2MATERIALS AND METHODSa) Animals

Albino rats (Charle River Wistar Cobs) aged 15-16 days, bred in the laboratory, were used in all experiments. Mothers received a pellet diet (41 B Oxoid) *ad libitum*, and tap water containing chlordiazepoxide hydrochloride (Librium, Roche) 20 mg/l to reduce cannibalism.

The day on which the litter was born was called day 1, however for litters born after 5.30 p.m. the following day was regarded as day 1. On the day after birth the size of the litter was reduced to ten and a further reduction to eight took place on day seven.

b) Preparation of labelled protein and Polyvinylpyrrolidone

Bovine, sheep, human and rat IgG, which had been prepared by chromatographic techniques and which gave single bands when characterized by immunoelectrophoresis and immunodiffusion, were obtained from Miles Laboratories Inc. Human transferrin and Bovine serum albumin (essentially globulin free) were obtained from Sigma Chemical Co. Ltd. These proteins were labelled with ^{125}I by the electrolytic method of Rosa, Scassellati, Pennisi, Riccioni, Giagnoni and Giordani (1964) at a level of one atom of iodine per molecule.

The labelling was carried out in a platinum crucible which constituted the anode, the cathode being a platinum wire enclosed in a glass tube which was sealed off at the bottom with dialysis tubing. A reference calomel electrode was included, and the potential between this and the anode was monitored throughout the electrolysis. After electrolysis the labelled protein solution was applied to a Sephadex G50 (coarse) column and eluted with 0.15M phosphate buffered saline (PBS) at pH 7.4, the eluant was analysed for protein content and radioactivity. The fractions

containing the labelled protein were pooled and the little unbound iodine which remained was removed by dialysis against PBS (pH 7.4). The protein concentration was adjusted to give a final value of 5 mg/ml, and the level of unbound iodine in this preparation, as determined by TCA precipitation, was found to be less than 1%.

Polyvinylpyrrolidone (PVP K.60, Fluka A.G., molecular weight 160,000) was labelled with ^{125}I by two methods. Originally the polymer was iodinated according to the method of Gordon (1958) which involved the heating of the polymer- ^{125}I mixture. This method yielded a 20 mg/ml solution of PVP containing 0.25-0.50 μc $^{125}\text{I}/\text{ml}$. The alternative method, as outlined by Briner (1961) relied upon ultra-violet irradiation of the PVP- ^{125}I mixture to effect the iodination, and gave a similar yield to the previous method.

All the isotopes were obtained from the Radiochemical Centre, Amersham.

c) Counting equipment

The labelled samples were assayed for radioactivity by means of a Packard Auto-Gamma Spectrometer with an automatic IBM teletype print out. Materials for gamma counting were placed in stoppered gamma-tubes (Packard Instrument Co.) and loaded directly into the automatic sample changer. The gamma radiation was detected by a thallium-activated sodium iodide crystal situated in the central well of the spectrometer.

d) Operative Procedure

i) For injection into the proximal small intestine

Rats aged 15-16 days were operated on using sodium pentobarbitone anaesthesia. The anaesthetic dose was administered *intra peritoneally* and was equivalent to 0.03 mg of sodium pentobarbitone per gramm of body weight.

An incision was made in the ventral body wall; the pyloric end of the stomach was withdrawn and a ligature was tied at the pyloric sphincter. 0.2 ml of labelled protein was injected into the duodenum distal to the ligature. The stomach was returned to the body cavity and the caecum and a length of distal small intestine was withdrawn. In rats aged less than 21-22 days the ileum is yellow-brown in colour, whereas the proximal region is creamy-white (Morris, 1975). The ileum of animals aged 15-16 days was carefully withdrawn until the colour began to change to creamy-white, and a second ligature was tied at this position. Thus the injected dose was confined to the proximal small intestine. The intestines were returned to the body cavity, and the body wall was sutured with silk thread.

At various time intervals after injection of protein into the proximal small intestine, the animals were killed, and that portion of the intestine which had received the radioactive protein was removed and washed out with 5 ml of cold phosphate buffered saline (PBS) at pH 6.2. Both the gut wash and the intestinal wall were subsequently assayed for radioactivity. A blood sample was collected from the heart in order to monitor the radioactivity present in the serum. The presence of small fragments of IgG in the serum was estimated by precipitating 0.1 ml aliquots of serum with ten volumes of 10% trichloroacetic acid (TCA), centrifuging for 15 minutes at 6,500g and counting the non-TCA-precipitable radioactivity in the supernatant.

ii) For injection into the distal small intestine

The ventral body wall was opened by a posteriorly placed incision and the caecum and a length of the distal small intestine were withdrawn from the body cavity. A ligature was tied at a position where the colour of the intestine began to change from yellow-brown to creamy-white, as described previously, and 0.2 ml of the labelled protein was injected

into the lumen distal to the ligature. A second ligature was then tied at the ileocaecal junction, thus enclosing the radioactive dose in a segment of the distal small intestine which had an unstretched length of 11-14 cm.

e) Determination of the distribution of radioactivity in the body

After intraluminal injection of labelled protein into the intestine the distribution of radioactivity in the various tissues of the animal was determined as follows:

$$I - R_t = R_e$$

where,

I = radioactivity of the intraluminally injected protein.

R_t = radioactivity remaining in the gut wash plus the gut wall after time t.

R_e = radioactivity removed from the intestine after time t.

The total radioactivity present in the vascular space was determined as follows:

$$V = S \times P$$

where,

V = the total radioactivity in the vascular space

S = the radioactivity of 1.0 ml of serum

P = the plasma volume of the animal.

The presence of small fragments of labelled protein in the serum was estimated by precipitating 0.1 ml aliquots of serum with ten volumes of 10% trichloroacetic acid (TCA), centrifuging for 15 minutes at 6,500 g and counting the non-TCA-precipitable radioactivity in the supernatant.

Throughout the duration of the experiment considerable amounts of radioactivity pass into the viscera and carcass from the vascular space. This amount was assessed thus;

$$R_e - V = C$$

where C = the radioactivity in the viscera and carcass.

The radioactivity present in the various tissues of the animal are expressed in μg equivalents of protein. This was determined by dividing the radioactivity present in a certain tissue by the radioactivity per μg of the intraluminally injected protein, for example;

$$\text{specific radioactivity of the injected protein} = \frac{10,000 \text{ c.p.m.}}{\mu\text{g}}$$

Radioactivity remaining in the gut = 2,000,000 c.p.m. therefore this represents $\frac{2,000,000 \text{ c.p.m.}}{10,000 \text{ c.p.m. } \mu\text{g}} \cdot 1 = 200 \mu\text{g}$ of protein.

This method has the advantage that direct comparisons can be made between experiments that involve different concentrations or different specific activities of the injected protein. The one reservation being that when μg equivalents are quoted for TCA-soluble radioactivity, this is not intended to represent μg 's of intact protein, but rather small breakdown products.

f) Ultracentrifugation

A variety of both preparative and analytical ultracentrifugation techniques were performed using a Beckman L5 65 B ultracentrifuge, incorporating a slow start accessory and an $\omega^2 t$ integrator.

Both differential and rate zonal methods were employed for the attempted purification of certain subcellular components such as lysosomes and coated vesicles.

Rate zonal ultracentrifugation was performed on linear sucrose density gradients prepared by means of an automatic gradient former. For better separation of the samples on preformed continuous gradients the starting and ending sucrose concentrations were made to differ by more than a factor of three, especially when the starting concentration was less than 10% (W/W).

After centrifugation the gradients were separated into four drop fractions by means of an LKB ULTRORAC fraction collection system with a flow cell Uvicord attachment which monitored the absorbance of the fractions at 280 nm. The radioactivity of each fraction was assayed and plotted, and the sucrose concentration of each separated zone was ascertained by using a hand refractometer. The sedimentation coefficients of the separated components were estimated by the method of McEwen (1967).

All ultracentrifugation procedures were performed at 4°C.

CHAPTER 3DETERMINATION OF PLASMA VOLUMEINTRODUCTION

In experiments involving the transmission of labelled immunoglobulins or other labelled proteins across the gut, it was necessary to estimate the amount of radioactivity that had been transported to the circulation. If the total volume of plasma in an animal is known, then the total radioactivity present in the circulation can be calculated as follows by assaying a small sample of serum for radioactivity;

$$R = r \left[\frac{V_p}{V_s} \right]$$

Where, R = total radioactivity in the plasma

r = radioactivity of the serum sample

V_p = plasma volume

V_s = volume of the serum sample

The values quoted for the plasma volume of young rats appear to vary with the age, weight and strain of the rats examined (Belcher and Harriss, 1957; Travnickova and Heller 1963; and Morris and Morris 1976). Consequently it was considered necessary to determine experimentally the value for the plasma volume of the rats that were being used in this particular study.

Methods

A radioactive tracer dilution method was used to determine the plasma volume of 15-16 day old rats in the 25-40g weight range. A 20 μ l dose of labelled homologous IgG or labelled PVP was injected into the heart using an Agla micrometer syringe (Wellcome). The head of the animal was monitored for radioactivity and a successful intra-cardiac injection resulted in an almost immediate increase in the radioactivity of the head. After a ten minute mixing period to allow a uniform distribution

of the tracer throughout the circulatory system, the animals were killed. At autopsy, blood was collected from the heart for haematocrit determination and the radioactivity of three 0.1 ml serum samples taken from this blood were measured.

The effects of larger doses of tracer and longer mixing times on the value of the plasma volume were also examined.

Results

According to simple isotope dilution theory, an unknown volume (V_u) can be calculated if the activity (A_s) and the volume (V_s) of the injected isotopic material and the activity (A_u) of the diluted isotopic material are known. This may be represented as follows:

$$V_u = V_s \left[\frac{A_s}{A_u} - 1 \right]$$

The radioactivity of three 0.1 ml aliquots of serum taken from 15-16 day old rats were assayed ten minutes after intracardiac injection of 20 μ l of ^{125}I labelled rat IgG, to determine plasma volume by dilution analysis. The plasma volume per 100g body weight, calculated for groups of animals of different weight ranges, is shown in table 3.1. The values obtained for the groups were not significantly different from one another and the overall mean was 6.55 ml/100g \pm 0.09 (29).

Table 3.2 shows for comparative purposes a combination of longer mixing times and larger doses. These results also include the values obtained from the use of an alternative radioactive tracer, PVP (molecular weight 160,000) which had been labelled with ^{125}I by two methods. These values show no significant differences from the previously calculated mean of 6.55 ml/100g. Taking an overall mean from all the data in tables 3.1 and 3.2, the plasma volume was estimated to be 6.57 ml/100g \pm 0.05 (57), and it is this value which has been used in all subsequent calculations.

Haematocrits on blood taken from the hearts of these rats were determined and gave a mean value of 29.16% \pm 0.52 (22). The blood volume

Table 3.1

The plasma volume of rats aged 15-16 days determined by dilution analysis using ^{125}I labelled rat IgG (20 μl) as the tracer. The results are given as mean values with the standard error of the mean (SEM) and with the number of samples in parentheses.

<u>WEIGHT RANGE</u>	<u>MEAN WEIGHT</u>	<u>PLASMA VOLUME (ml/100g BODY WEIGHT)</u>
26.0-30.0	27.7 ± 0.41	6.54 ± 0.17 (9)
30.1-35.0	32.3 ± 0.35	6.43 ± 0.15 (12)
35.1-40.0	36.9 ± 0.47	6.75 ± 0.12 (8)
OVERALL MEAN		6.55 ± 0.09 (29)

Table 3.2

The plasma volume of 15-16 day rats as determined by dilution analysis using ^{125}I rat IgG and ^{125}I PVP as tracers. A comparison between various mixing times, tracers and dose volumes. The results are given as mean values \pm SEM and with the number of samples in parentheses.

TRACER	DOSE (μl)	MIXING TIME (min.)	PLASMA VOLUME (ml/100g BODY WEIGHT)
RAT IgG	50	20	6.58 \pm 0.09 (3)
RAT IgG	100	10	6.69 \pm 0.09 (5)
RAT IgG	100	30	6.69 \pm 0.09 (10)
PVP ₁	20	10	6.57 \pm 0.10 (3)
PVP ₂	20	10	6.43 \pm 0.08 (7)

1. P.V.P. iodinated by the method of Gordon (1958)
2. P.V.P. iodinated by the method of Briner (1961)

calculated from these mean figures was 9.27 ml/100g body weight.

Discussion

In these investigations, a radioactive tracer dilution method was used to determine the plasma volume of neonatal rats in the 25-40g weight range. The major difficulty in the dilution method is the need to allow sufficient time for uniform mixing of the tracer, during which time some may be lost from the vascular space. Furthermore, adequate time should elapse after injection to allow the homeostatic re-establishment of the previous blood volume. However in these studies owing to the very small volume of the injected tracer, interference caused by this factor was regarded as minimal.

There are various methods available employing the dilution principle using either labelled red blood cells or substances which travel with the plasma. In the past several radio-isotopes have been used as specific erythrocyte labels such as ^{55}Fe (Hahn *et al* 1941), ^{59}Fe (Gibson 1946), ^{51}Cr (Gray *et al* 1950), ^{32}P (Hahn *et al* 1940) and ^{42}K (Yallow 1951). The most commonly used plasma soluble substances have been Evans blue dye T-1824, and more recently plasma proteins tagged with ^{131}I and ^{125}I (Storaasli *et al* 1950; Krieger *et al* 1948 and Crispell 1950). Studies on the comparability of these methods revealed that Evans blue dye and iodinated proteins produce no significant differences in the value obtained for the plasma volume (Schultz *et al* 1953; and Crispell *et al* 1950). However these plasma soluble markers did result in higher values for the blood space than similar experiments using tagged red cells (Berson *et al* 1952). This may be due to the plasma proteins being distributed throughout the vascular space and part of the lymphatic system, so that the measured space may be slightly larger than the volume calculated by the red cell method since red cells do not cross capillary membranes. Although the red cell method may seem a more precise measure of the blood volume, the need to label the animal's erythrocytes introduces many practical disadvantages

over the plasma protein method. Consequently it was decided to use rat IgG as the vehicle to carry the ^{125}I label.

In order to examine whether or not catabolism of the labelled IgG over the mixing period was significant, P.V.P. was substituted for IgG as the ^{125}I carrier molecule since it is known that this polymer does not undergo catabolism. The polymer was iodinated by two methods for comparative purposes, and as shown in table 3.2, neither tracer produced values for the plasma volume that were significantly different to the value obtained by using IgG. As an additional check, the IgG mixing time was extended from 10 to 30 minutes, and this did not produce an increase in the estimate of plasma volume. Consequently the clearance of IgG from the plasma over the mixing period was considered to be negligible.

Using these methods a value for the plasma volume of neonatal rats in the 25-40g weight range was calculated to be 6.57 ml/100g body weight. This value is greater than that recorded by Belcher and Harriss (1957) who used ^{131}I -labelled human albumin as a marker, and reported a serum volume of 5.38 ml/100g ± 0.27 for brown hooded rats of an August strain with body weights in the 26-50g range. Morris and Morris (1976), using slightly lighter rats of a Wistar strain, reported a value of 5.53 ml/100g ± 0.15 , with rat IgG as the labelled protein. A similar value of 5.40 ml/100g ± 0.05 was produced by Travnickova and Heller (1963) by means of an Evans blue dye method. These values all appear to be lower than the value quoted in this study, which would suggest that the ^{125}I IgG was being cleared rapidly from the circulation, so producing anomalously high results. However this possibility was examined by the use of ^{125}I PVP, which is not catabolised, and by increasing the mixing time for IgG up to 30 minutes. Neither factor led to an alteration in the estimation of plasma volume, and consequently the value of 6.57 ml/100g was considered to be valid.

Discrepancies between this and previous studies on the plasma volume

may be due to differences in the body weights, ages, strains and diets of the animals used in these studies.

CHAPTER 4THE UPTAKE AND TRANSMISSION OF PROTEINS BY NEONATAL RAT ENTEROCYTESIntroduction

Intact homologous IgG is transmitted to the circulation through the enterocytes of the proximal region of the small intestine of suckling rats (Morris and Morris 1976). The passage of intact molecules through the cells is said to involve attachment to receptors at the cell surface, which bind specifically to the Fc portion of the IgG molecule (Waldman and Jones 1973; Rodewald 1973). It has been suggested that the binding of IgG to the receptor protects the molecule from digestion during passage through the enterocyte. The experiments reported in this chapter were designed to compare the transmission of heterologous and homologous immunoglobulins and other protein molecules, thus comparing the degree of protection afforded to these various proteins. Investigation of the breakdown products produced by the digestive activity of the gut has also been carried out and the rates at which proteins and breakdown products were removed from the vascular compartment have been assessed.

MethodsOperative procedure

Groups of eight animals were operated on as described in Materials and Methods, and 0.2 ml of the preparation containing 1 mg of labelled protein was injected into the duodenum. Two hours after injection the animals were killed and a blood sample collected from the heart. The left iliac vein was severed and the body was perfused by pumping phosphate buffered saline (pH 6.5) into the heart. Perfusion continued for about ten minutes. The perfusates were pooled, centrifuged to remove red cells, and the supernatant collected for ultrafiltration. The radioactivity in 0.1 ml aliquots of serum was assayed, and the percentage that was TCA precipitable determined.

The portion of the intestine which had received the radioactive protein was removed, washed out with 5 ml of cold PBS (pH 6.5), and the gut wash and gut wall were assayed for radioactivity. The viscera from four animals were pooled and homogenized in about 25 ml of PBS; and the carcasses were homogenized in about 50 ml of PBS. The viscera and carcass homogenates were centrifuged at 4°C at 20,000g for two hours (70 Ti rotor in a Beckman L5 65 B ultracentrifuge). The carcass supernatant was passed through a coarse filter and the filtrate was retained for ultrafiltration. The viscera supernatant did not usually require this treatment prior to ultrafiltration. Sodium azide was added to samples to give a final concentration of 0.05% to act as a bacteria-stat, except for those samples which were to be re-injected into animals.

Interference studies

The capacity of various unlabelled heterologous IgGs and proteins to interfere with the uptake of labelled rat IgG from the neonatal rat gut was examined. 0.2 ml of labelled rat IgG containing 1 mg of labelled and 1 mg of unlabelled protein was injected into the ligated proximal small intestine. Three hours after injection the animals were operated upon, and a blood sample collected from the heart. The distribution of radioactive material in the intestines, vascular compartment and the carcass was determined as described previously.

Ultrafiltration

Ultrafiltration of the perfusates and supernatants from the viscera and carcass homogenates was carried out using a Chemlab C 50 ultrafiltration cell (50 ml capacity) employing a pressure of 30 p.s.i. of nitrogen. Material was passed through the following Amicon Diaflow ultrafiltration membranes (43 mm diameter) to leave a residual solution of 3-5 ml;

>100,000 mol. wt (XM 100A); >50,000 mol. wt. (XM 50); >1,000 mol. wt. (UM 2). At each step the volumes of concentrate and filtrate were measured and their radioactivity assayed, which enabled the amount of radioactive material in the successive concentrates and filtrates to be calculated.

Density Gradient Ultracentrifugation

Rate zonal ultracentrifugation was performed on 0.3 ml samples of serum from animals two hours after the intraluminal injection of 0.2 ml of ^{125}I labelled protein. The serum samples were applied to the top of linear 5-25% (w/w) sucrose density gradients, in pollyallomer tubes of 13.2 ml capacity. These gradients were centrifuged for 34 hours at 205,600g (average) at 4°C (SW41Ti rotor). After centrifugation the gradients were fractionated as described in Materials and Methods.

Intra-cardiac injection

A 0.1 ml dose of a solution containing either labelled IgG (bovine, sheep or human) or labelled bovine serum albumin or human transferrin, was injected into the heart of 15-16 day old animals. The animals were killed after 30 minutes, one hour, two hour and three hour intervals, and at autopsy blood was collected from the heart and the serum radioactivity was determined.

The perfusates, obtained from animals which had received labelled preparations into the proximal small intestine, were concentrated by ultrafiltration, and 0.1 ml of these concentrates were injected intra-cardiac. The serum was assayed for radioactivity after the same time intervals as above.

Digestion of labelled proteins

The lumina of the proximal regions of the small intestine of fourteen 16 day old rats were washed out with 5 ml of cold PBS (pH 6.5) from a 20 ml pool. 25 μ l aliquots of the labelled proteins, at a concentration of 5 mg/ml were added to 475 μ l aliquots of the gut washes and controls in polystyrene centrifuge tubes. Tubes were incubated at 37 $^{\circ}$ C for either two or four hours. After incubation, 100 μ l of BSA solution (10 mg/ml) and 1.4 ml of 10% TCA were added to each tube and the radioactivity was assayed. The tubes were then centrifuged for 45 minutes at 3,500 g and aliquots of the supernatants were removed and the radioactivity assayed to determine the non-TCA-precipitable activity present after incubation.

RESULTS

Removal of labelled protein from the intestine

Young rats aged 15-16 days received injections of labelled protein (1,000 μ g) into the ligated proximal small intestine and two hours later were killed. The radioactivity remaining in the intestine was measured and the amount which had been removed was determined by subtraction. The radioactivity of the serum was assayed and the amount present in the vascular compartment was calculated. The distribution of radioactive material throughout the bodies of the animals was determined as outlined in Materials and Methods. These results are shown in Table 4.1, and are expressed in μ g equivalents of protein.

Analysis of the data by means of a studentized range test (Scheffé 1959) revealed that there was no significant difference at the 5% level between the amount of rat, human, sheep or bovine IgG remaining in the gut wash. The gut wash value for BSA was not significantly lower than for human IgG, however it was significantly lower than the other IgG's. The amount of human transferrin in the gut wash appeared to be significantly

Table 4.1

The radioactivity present in the vascular compartment and the viscera plus carcass two hours after the injection of 1mg of labelled protein into the ligated proximal small intestine. Radioactivity is expressed as μg equivalents of labelled protein. The results are presented as the mean \pm S.E.M. for groups of eight animals.

Protein Injected	Radioactivity remaining in the gut wash	Radioactivity remaining in the gut wall	Radioactivity removed from the gut	Radioactivity in the vascular compartment TCA precipitable	Radioactivity in the viscera and carcass
Rat IgG	176.5 \pm 16.2	350.6 \pm 12.9	472.9 \pm 19.3	187.3 \pm 13.3	271.7 \pm 9.8
Human IgG	135.5 \pm 10.2	384.3 \pm 10.5	480.2 \pm 10.1	163.5 \pm 8.2	300.1 \pm 13.9
Sheep IgG	169.3 \pm 16.6	271.8 \pm 22.0	558.9 \pm 23.0	102.2 \pm 8.1	435.9 \pm 29.0
Bovine IgG	177.9 \pm 15.2	239.0 \pm 18.0	583.1 \pm 24.4	76.3 \pm 7.6	478.6 \pm 30.0
BSA	104.2 \pm 27.6	140.2 \pm 31.5	755.6 \pm 31.8	26.2 \pm 4.2	683.9 \pm 31.8
Human Transferrin	50.7 \pm 6.2	43.4 \pm 4.5	905.9 \pm 6.9	15.9 \pm 1.8	835.5 \pm 12.2

less than any other injected protein.

Two hours after the intraluminal injection of rat and human IgG, there was no significant difference between the amount of radioactivity remaining in the gut wall. Similarly there was no significant difference between the amount of bovine or sheep IgG remaining in the gut wall. However the differences between the sheep and bovine pair and the human and rat pair are significant.

The mean amount of the dose removed from the intestine in animals which had received bovine IgG was 583 μg and was not significantly different from that removed after the injection of sheep IgG (559 μg). The mean values for the human IgG group (480 μg) and the rat IgG group (473 μg) were both significantly lower than the values for the sheep and bovine IgG groups.

The total radioactivity in the vascular compartment, after the injection of labelled protein into the proximal small intestine, is made up of radioactive TCA-precipitable protein and non-TCA-precipitable radioactive fragments. The radioactivity in the vascular compartment was very high after the injection of rat IgG (about 20% of the dose) and human IgG (about 18% of the dose), and in both cases more than 90% of the activity was TCA-precipitable. The total radioactivity in the vascular compartment was significantly lower after the injection of other proteins. About 83% of the radioactivity was TCA-precipitable after the injection of sheep IgG; about 72% with bovine IgG, about 36% with BSA and 23% with transferrin.

The radioactive sera were fractionated after ultracentrifugation in sucrose density gradients. The serum collected from animals after the injection of IgG showed large peaks of radioactivity at 7s, similar to the peak obtained in runs with intact, labelled IgG. After the injection of transferrin and BSA the radioactivity of the serum was relatively low,

and consisted mainly of low molecular weight breakdown products, (see Fig. 4.1 - 4.8).

The mean radioactivity in the viscera and carcasses was very high after the injection of transferrin, significantly greater than after the injection of BSA. The viscera and carcass values for both these proteins were significantly greater than after immunoglobulin injection. There was no significant difference between the viscera and carcass values for the bovine and sheep groups, but the values for these groups were both significantly greater than either the rat or the human groups.

Interference Studies

The results in table 4.2 and Fig. 4.9 clearly demonstrate that the capacity of one immunoglobulin molecule to interfere with the intact transmission of another immunoglobulin depends upon the species of origin of the former. It can also be seen from this data that non-immunoglobulin molecules such as BSA and transferrin have no ability to interfere with the process of intact immunoglobulin transmission from the neonatal gut.

Ultrafiltration

The perfusates and the supernatants of the viscera and carcass homogenates, from animals two hours after the injection of labelled protein into the proximal small intestine, were ultrafiltered. The results are shown in table 4.3.

After the intra-intestinal injection of immunoglobulins (molecular weight approximately 158,000), the radioactivity in the vascular compartment was either associated with material exceeding 100,000 mol.wt. or less than 1,000 mol.wt., with very little associated with material of intermediate size. Similar results were obtained with the viscera and carcass homogenates.

Fig. 4.1 The determination of the sedimentation coefficient (S value) of a labelled IgG standard by means of ultracentrifugation on a 5-25% (w/w) sucrose density gradient.

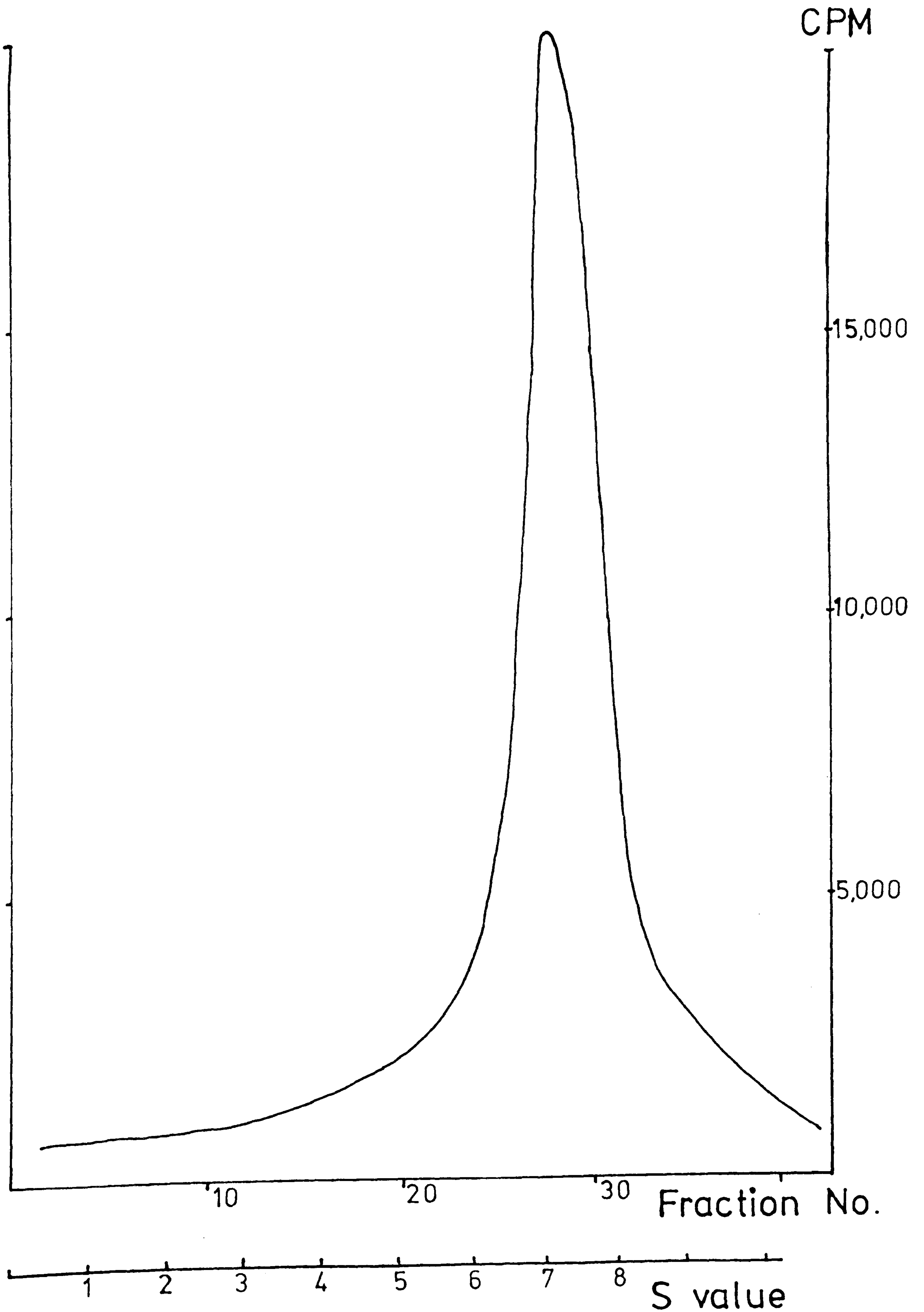


Fig. 4.2 The fractionation, on a 5-25% (w/w) sucrose density gradient, of a 0.3 ml serum sample obtained two hours after the injection of labelled rat IgG into the proximal small intestine of a 15-16 day old rat. The results show the radioactivity (cpm) and the sedimentation coefficient (S value) of the separated components.

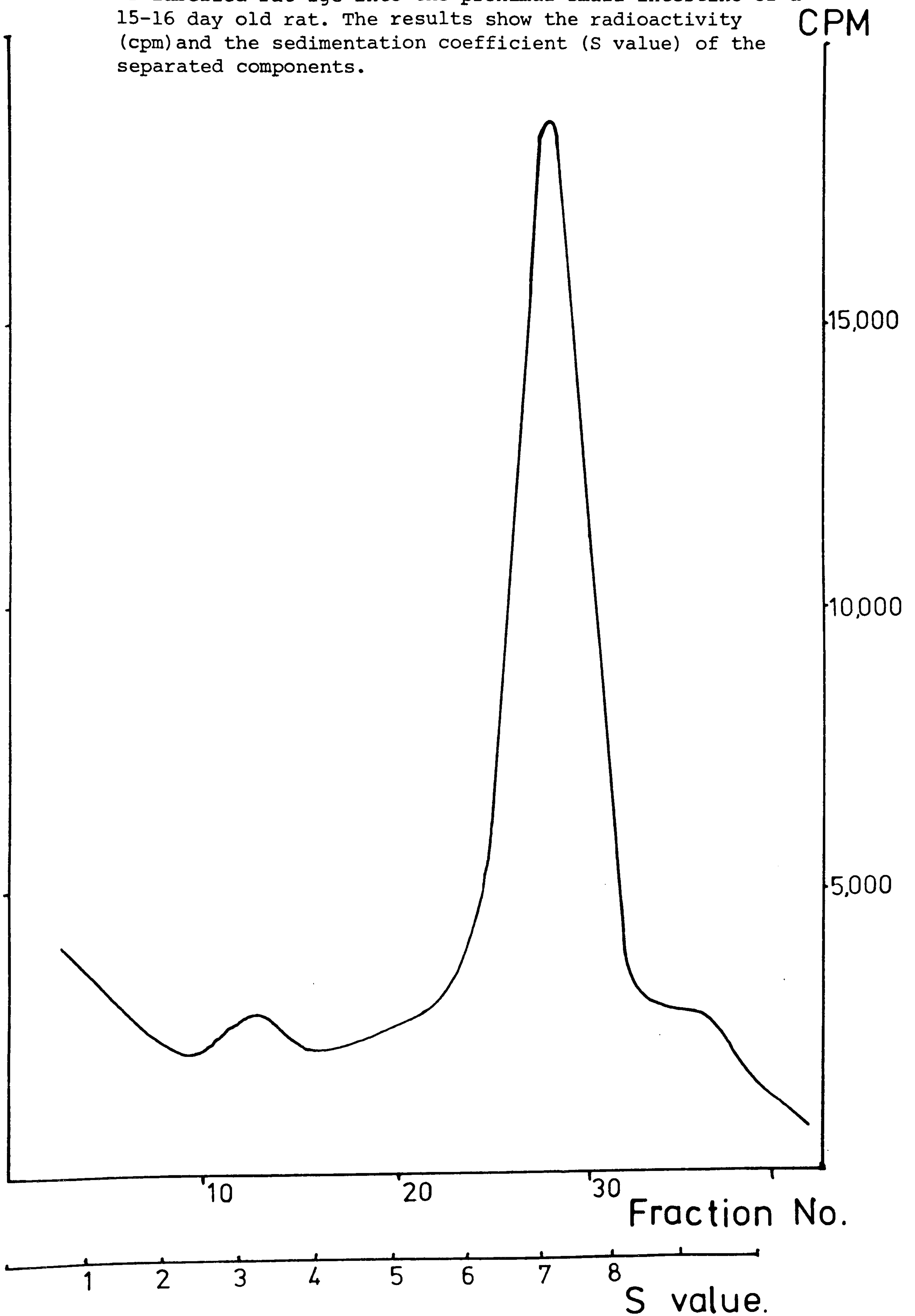


Fig. 4.3 The fractionation, on a 5-25% (w/w) sucrose density gradient, of a 0.3 ml serum sample obtained two hours after the injection of labelled human IgG into the proximal small intestine of a 15-16 day old rat. The results show the radioactivity (cpm) and the sedimentation coefficient (S value) of the separated components.

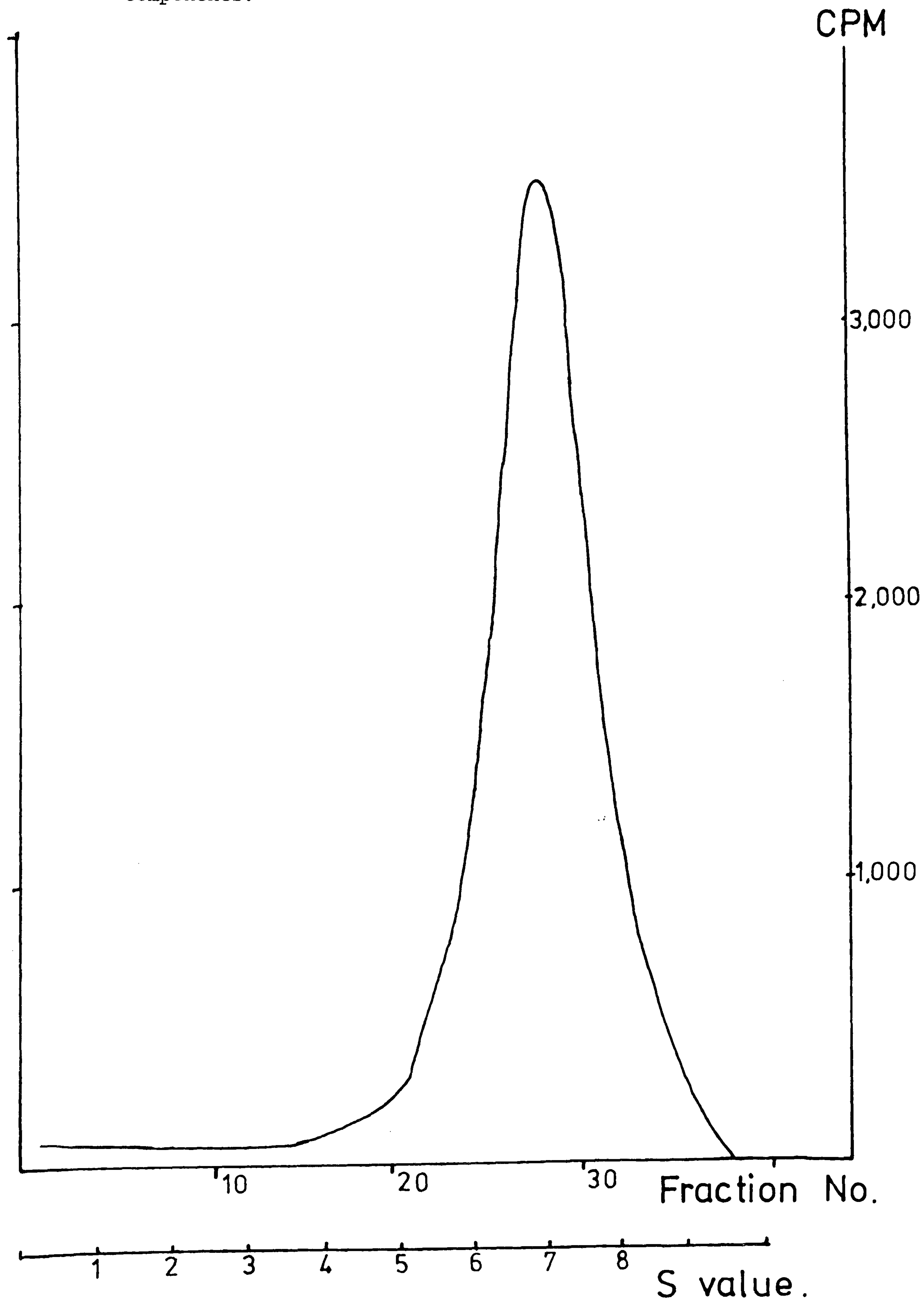


Fig. 4.4 The fractionation, on a 5-25% (w/w) sucrose density gradient, of a 0.3 ml serum sample obtained two hours after the injection of labelled sheep IgG into the proximal small intestine of 15-16 day old rats. The results show the radioactivity in c.p.m. (r), the % absorbtion at 280 nm (uv) and the sedimentation coefficient of the separated components.

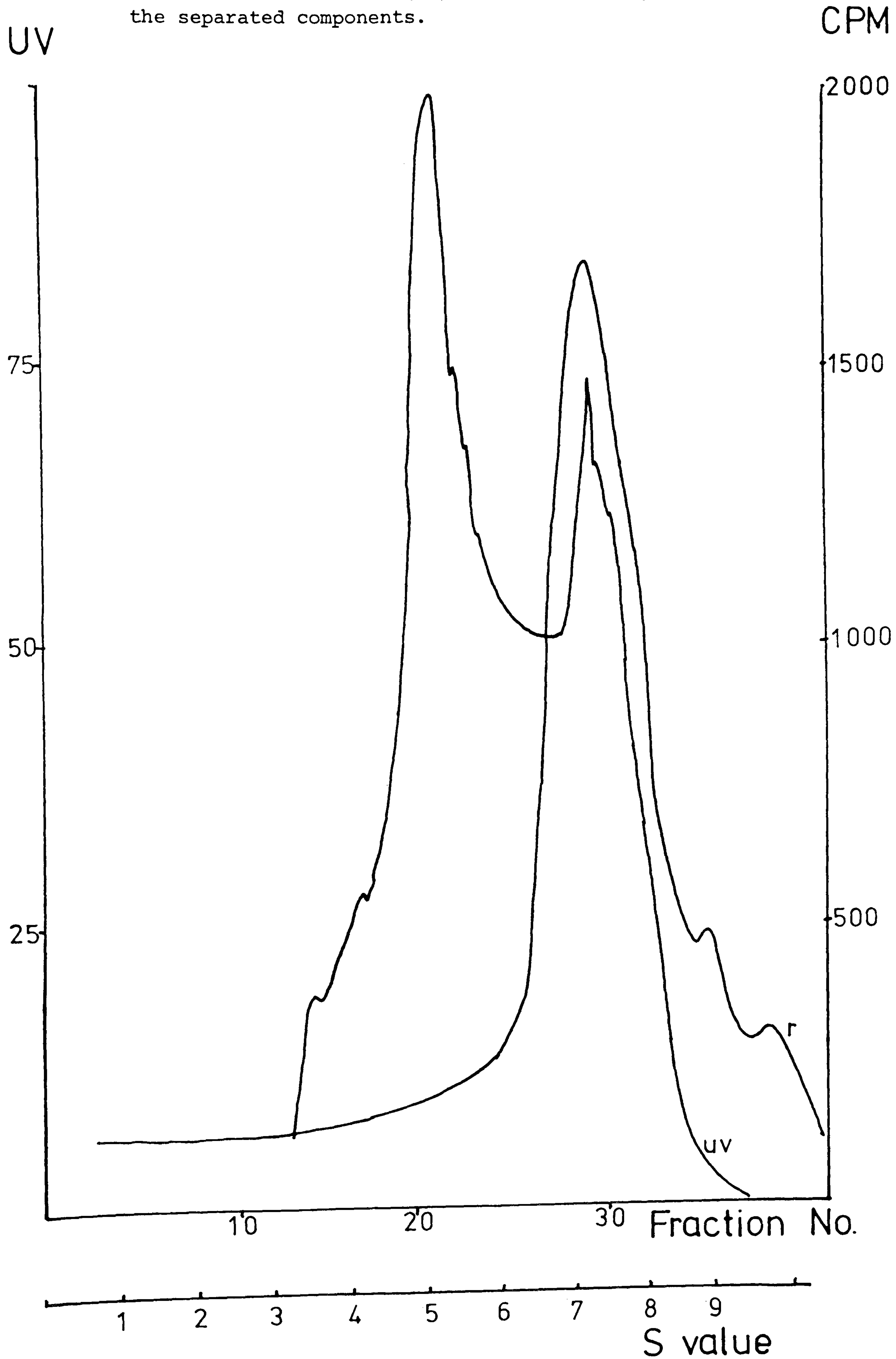


Fig. 4.5 The fractionation, on a 5-25% (w/w) sucrose density gradient, of a 0.3 ml serum sample obtained two hours after the injection of labelled Bovine IgG into the proximal small intestine of 15-16 day old rats. The results show the radioactivity in c.p.m. (r), the % absorbtion at 280 nm (uv) and the sedimentation coefficient of the separated components.

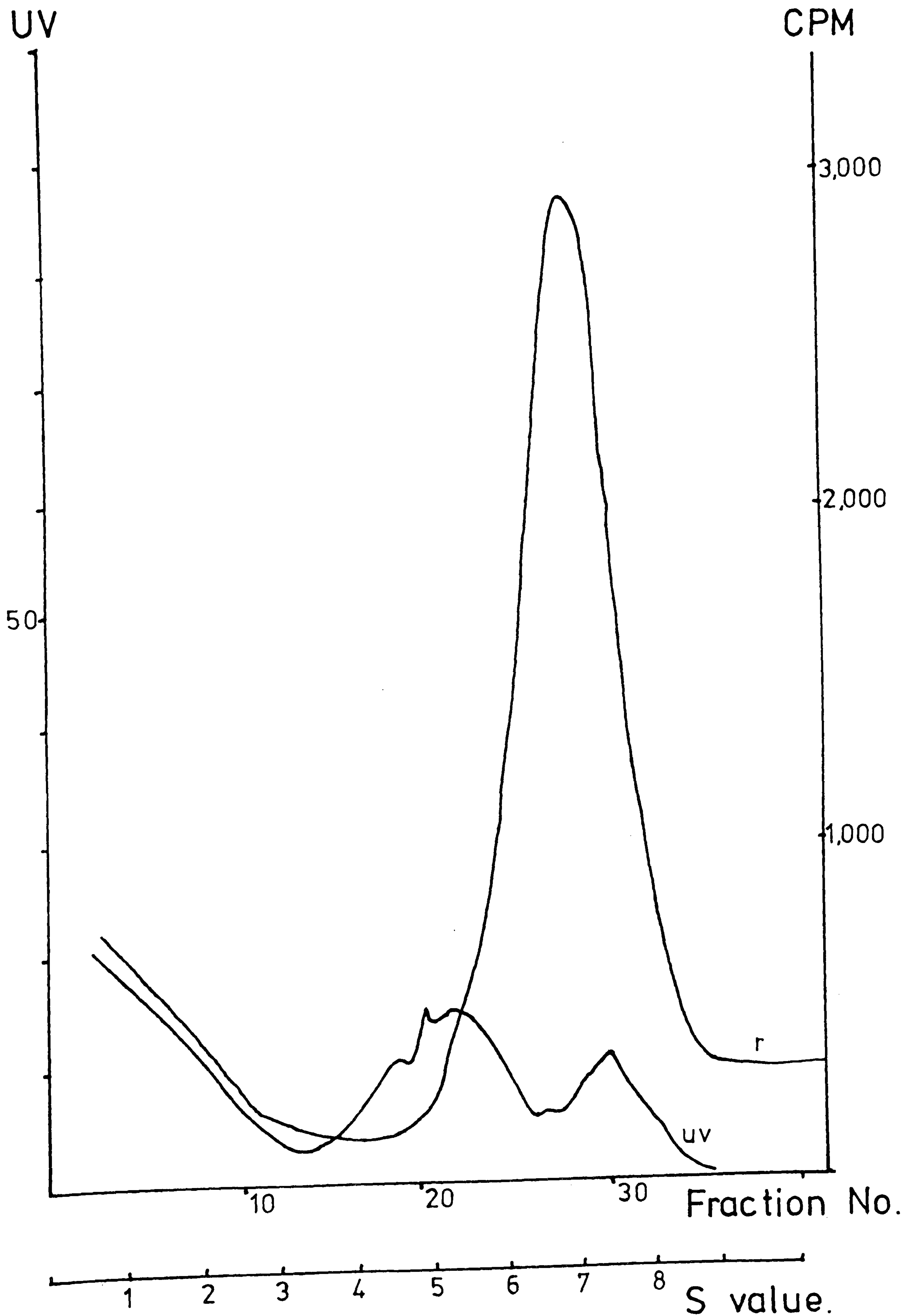


Fig. 4.6 The fractionation, on a 5-25% (w/w) sucrose density gradient, of a 0.3 ml serum sample obtained two hours after the injection of labelled Bovine serum albumin into the proximal small intestine of 15-16 day old rats. The results show the radioactivity in c.p.m. (r), the % absorbtion at 280 nm (uv) and the sedimentation coefficient of the separated components.

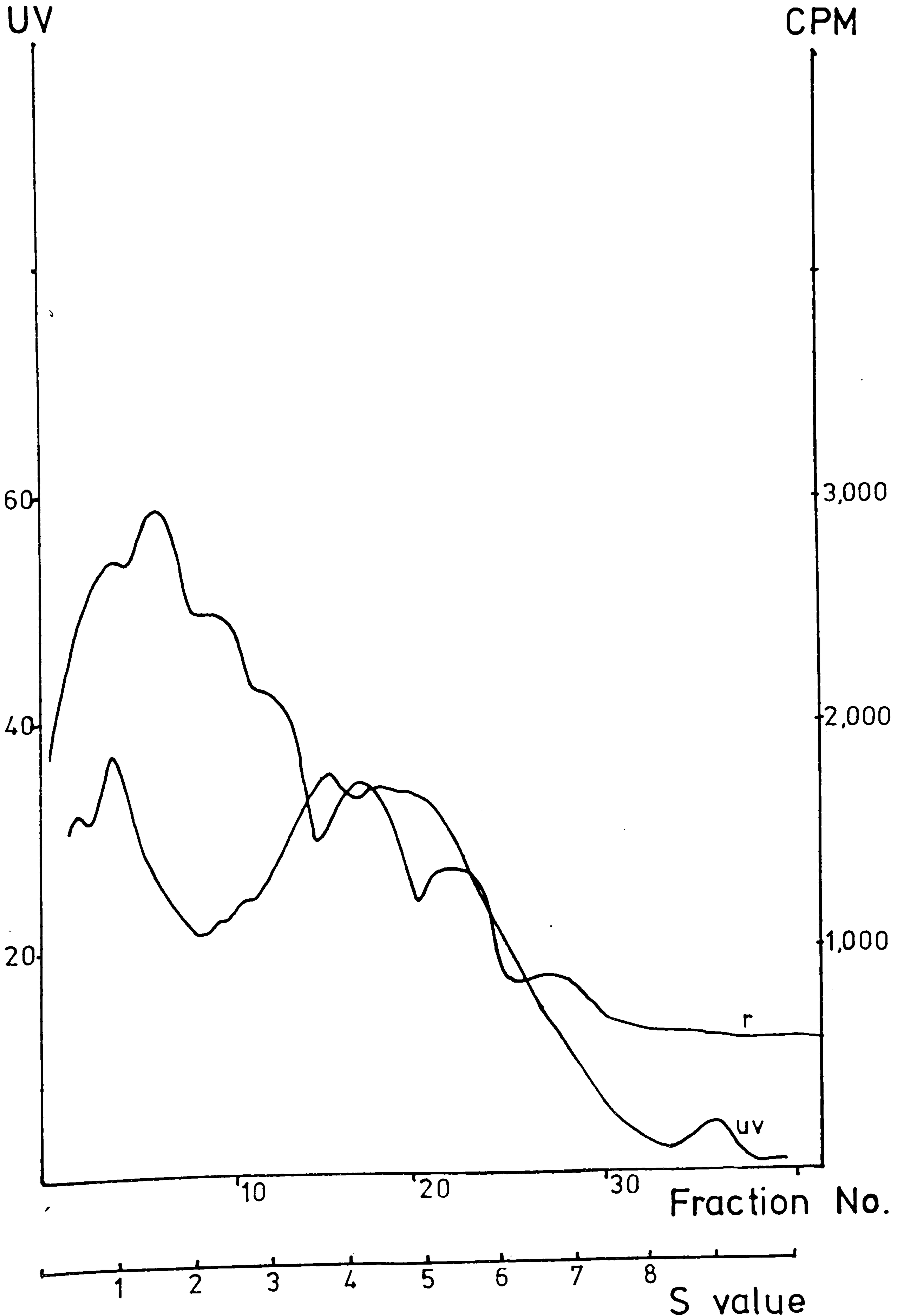


Fig. 4.7 The determination of the sedimentation coefficient (S value) of a labelled human transferrin standard by means of ultracentrifugation on a 5-25% (w/w) sucrose density gradient.

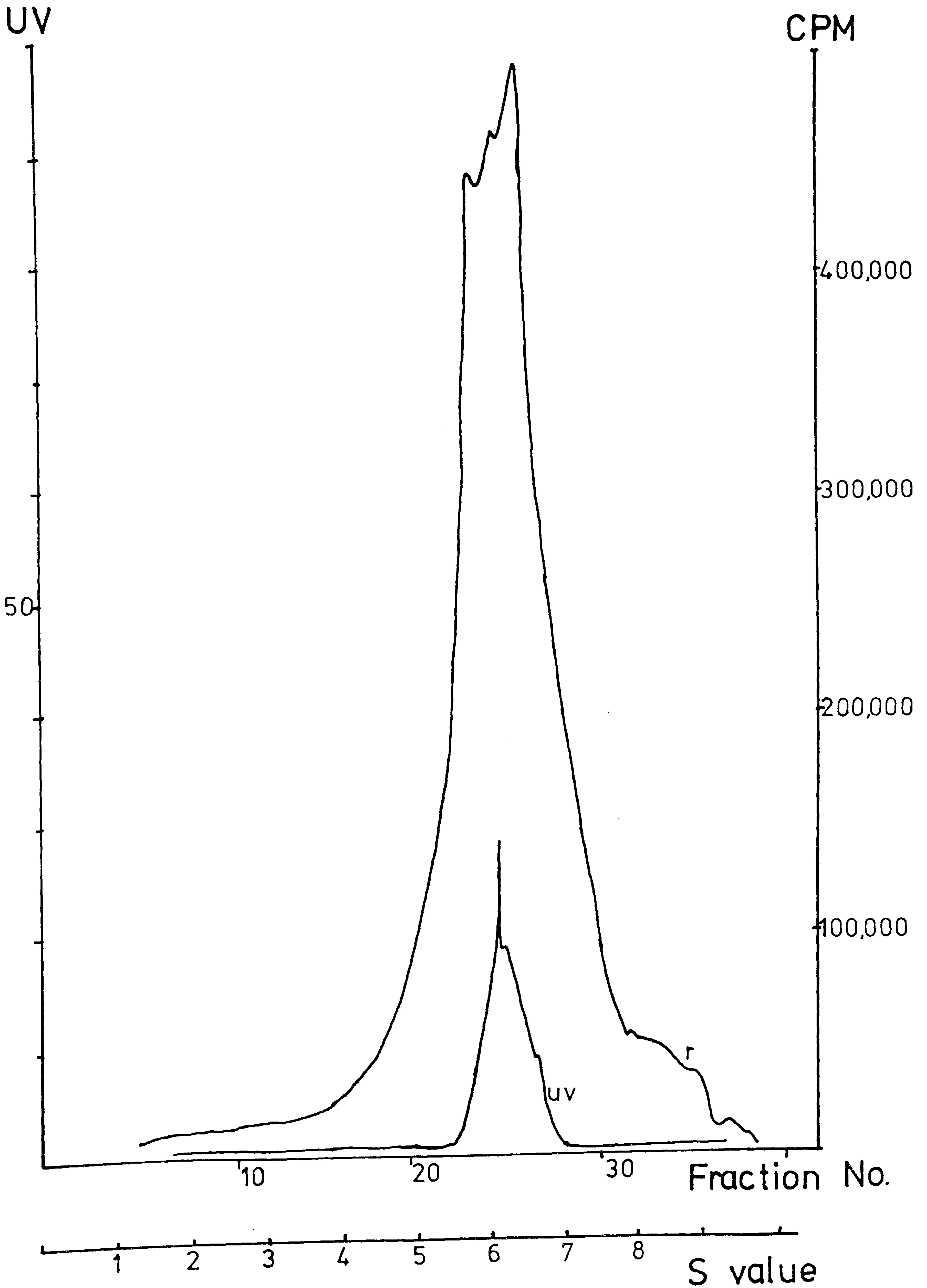


Fig. 4.8 The fractionation, on a 5-25% (w/w) sucrose density gradient, of a 0.3 ml serum sample obtained two hours after the injection of labelled Human Transferrin into the proximal small intestine of 15-16 day old rats. The results show the radioactivity in c.p.m. (r), the % absorbtion at 280nm (uv) and the sedimentation coefficient of the separated components.

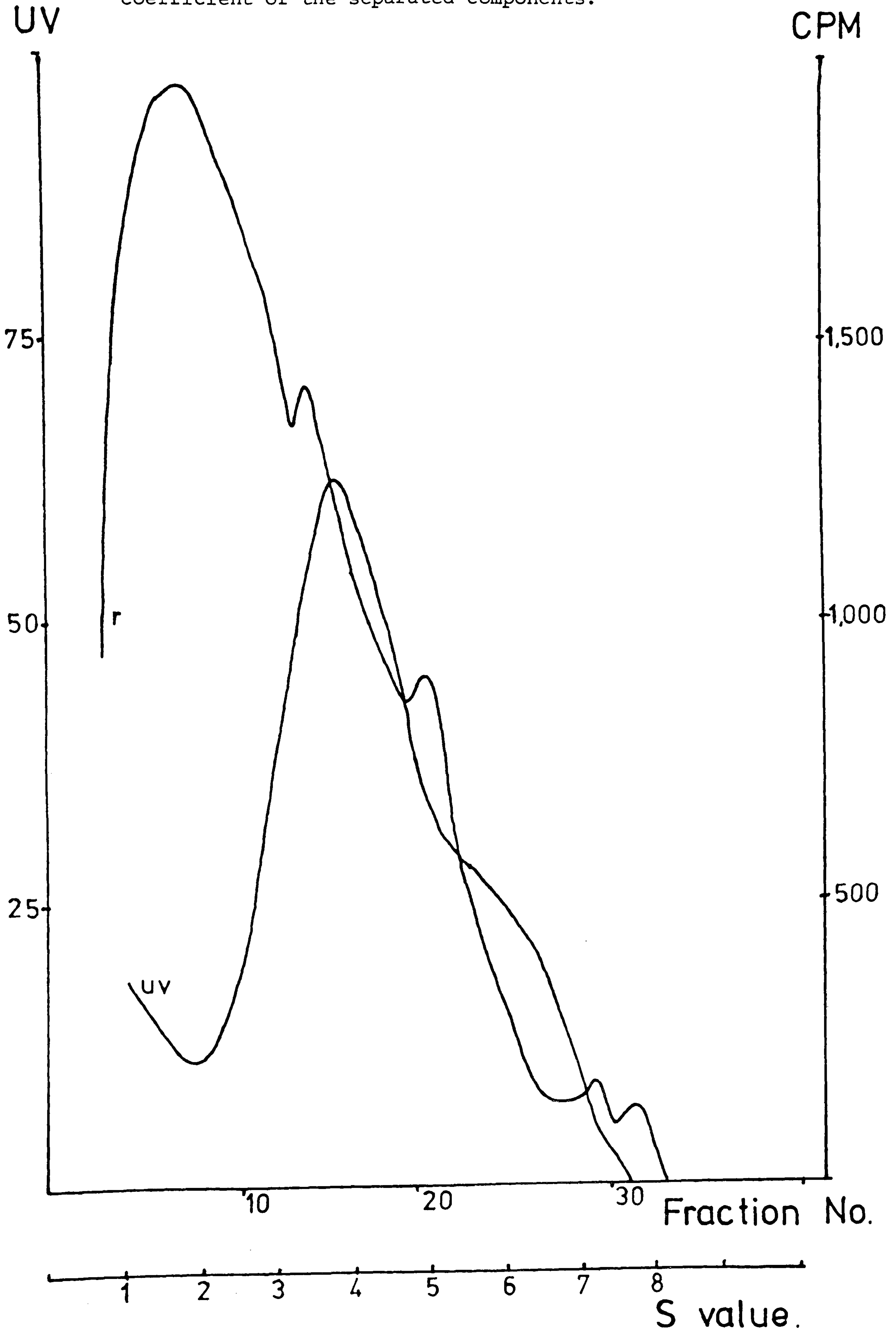
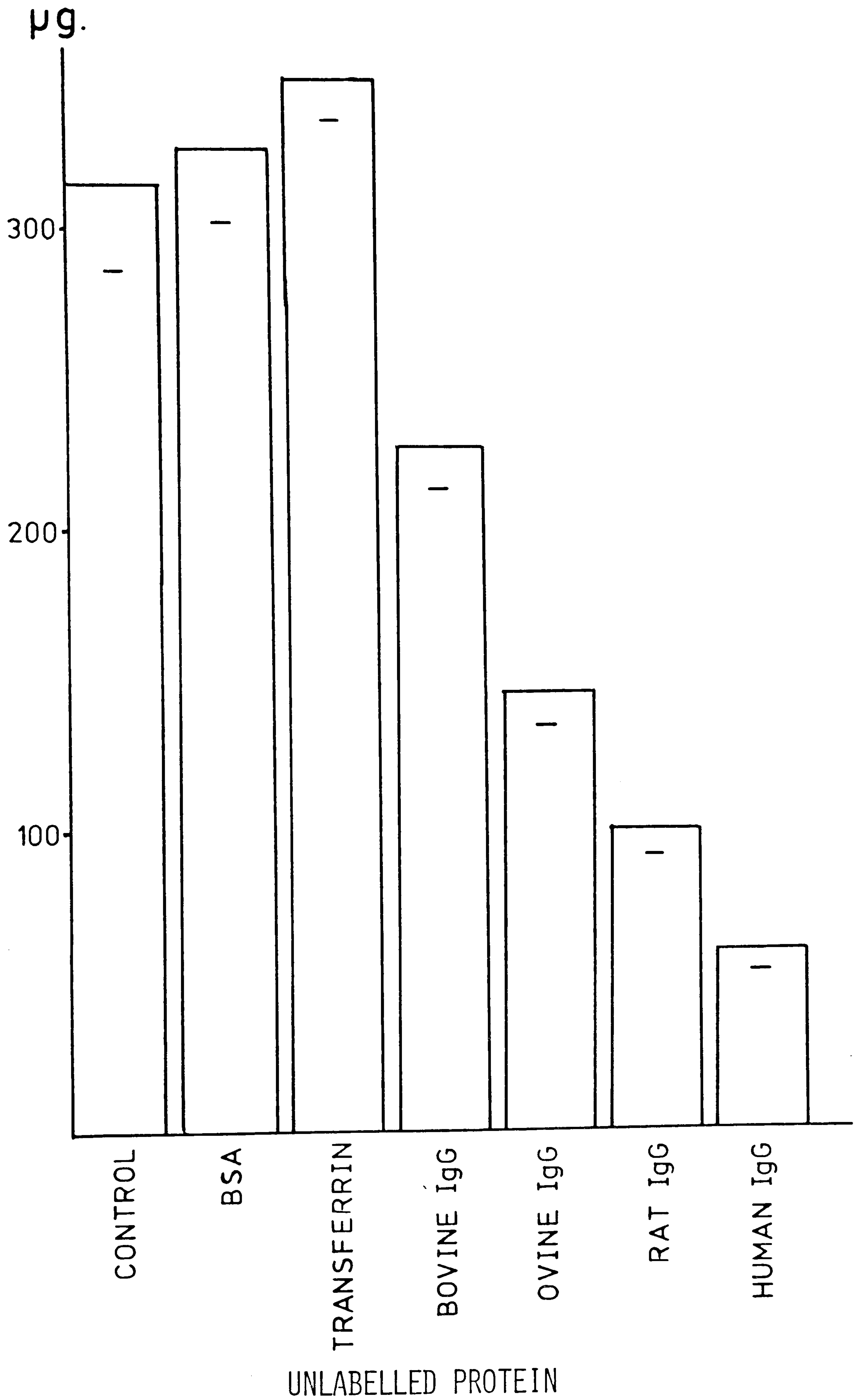


Table 4.2 The mean amount of TCA-precipitable radioactive material (given in μg) present in the vascular compartment of 15 day rats, three hours after the injection into the ligated proximal small intestine, of ^{125}I labelled rat IgG(1mg) in the presence of 1mg of unlabelled protein. The figures represent the means \pm S.E.M. and the number of samples are shown in parentheses.

UNLABELLED PROTEIN	μg OF TCA-PRECIPITABLE RADIOACTIVE MATERIAL IN THE VASCULAR COMPARTMENT
None (11)	315.0 \pm 23.2
BSA (5)	327.8 \pm 24.6
Transferrin (5)	351.3 \pm 13.8
Bovine IgG (5)	228.3 \pm 14.4
Ovine IgG (5)	146.5 \pm 11.7
Rat IgG (5)	100.6 \pm 8.2
Human IgG (5)	60.4 \pm 6.9

Fig. 4.9 The mean amount (\pm S.E.M.) of TCA-precipitable radioactive material (given in μ g) present in the vascular compartment, three hours after the injection of 1.0 mg of labelled rat IgG into the proximal small intestine, in the presence of 1.0 mg of unlabelled protein.



After the injection of human transferrin (mol. wt. 90,000) into the small intestine, all of the radioactivity in the perfusates and in the homogenates of the viscera and carcass passed through the 1,000 mol. wt. filter; however after the injection of BSA (mol. wt. 66,000) about 20% of the radioactivity of the perfusate, about 7% of that of the viscera and about 2% of that of the carcass was associated with molecules larger than 50,000 mol. wt. The samples ultrafiltered after BSA injection were those which had relatively larger amounts of TCA-precipitable radioactivity in the serum, and these were the samples used for intra-cardiac injection.

Relative digestibility of proteins

The relative digestibility of the labelled proteins used in these experiments was assessed by comparing the amounts of non-TCA-precipitable radioactivity which result after incubating the proteins with the gut wash from the proximal small intestine. The radioactivity which was not precipitated with TCA in control samples was subtracted from experimental values, and the non-precipitable radioactivity due to digestion was expressed as a percentage of the starting protein-bound activity. The results presented in table 4.4 show that the amount of transferrin digested after two and four hours was much greater than any other protein ($p < 0.001$); whereas BSA was relatively less digestible and was comparable in this respect with the various immunoglobulins.

Rates of clearance from the vascular compartment

Animals were killed half, one, two and three hours after the intra-cardiac injection of 0.1 ml. of the labelled preparations containing 500 μ g of protein, to determine the rate at which intact molecules left the vascular compartment. Perfusates obtained from animals, after the

injection of labelled proteins into the small intestine, were concentrated by ultrafiltration and the concentrates were injected, intra-cardiac, as above. In Fig. 4.10A and 4.10B the clearance rates for the various preparations and perfusates are plotted. The concentrates produced by ultrafiltration, using a 100,00 mol. wt. filter after IgG injection, or a 50,000 mol. wt. filter after transferrin and BSA injection, contained some small molecular weight components which would have passed the filter had filtration been continued. Such small molecular weight material left the vascular compartment rapidly. Consequently regression analysis of the results (Table 4.5) was based on 1, 2 and 3 hour values assuming a linear relationship over this period. The results were analysed using the method of least squares and the significance between regressions was analysed for differences of slope and intercept using an analysis of variance technique (Brownlee 1946).

The results presented in Fig. 4.10 and Table 4.5 show that the slopes for the regression lines, and hence the clearance rates for the heterologous IgGs are not significantly different from each other and are very similar to the rates calculated by Morris and Morris (1976) for rat IgG, which are included here for comparison. The slopes of the regression lines for BSA and transferrin were very similar to each other and were not significantly different from those obtained for the immunoglobulins. However, BSA and transferrin were cleared from the vascular space more rapidly than the immunoglobulins during the 0-1 hour period; so that the intercept values for these molecules were significantly different from those calculated for the immunoglobulins.

Ultrafiltrates had high initial clearance rates (0-1 hour) but thereafter the regression slopes for the perfusates were not significantly different from those obtained with the intact labelled proteins. The serum perfusates obtained after the intra-intestinal injection of BSA and

Table 4.3 The percentage of the radioactivity in the vascular compartments, and in the homogenates of the viscera and carcass, associated with different molecular weight material, 2 hr after the injection of labelled protein into the small intestine. Means and S.E. from three samples.

Protein injected into intestine	Mol. wt.	Vascular Compartment	Viscera	Carcass
Bovine IgG	>100,000	56.8 ±1.4	20.3 ±1.2	8.0 ±0.1
	< 1,000	43.0 ±1.4	79.5 ±0.9	91.8 ±0.1
Sheep IgG	>100,000	60.0 ±2.5	38.0 ±0.8	21.5 ±1.9
	< 1,000	39.0 ±2.3	59.5 ±0.8	76.6 ±1.7
Human IgG	>100,000	69.0 ±1.3	38.7 ±1.2	20.0 ±1.0
	< 1,000	28.9 ±1.4	59.9 ±1.1	77.0 ±1.2
Rat IgG	>100,000	83.2 ±1.8	44.9 ±7.8	19.7 ±3.4
	< 1,000	15.3 ±2.6	55.0 ±8.3	80.0 ±6.4
Transferrin	> 50,000	nil	nil	nil
	< 1,000	100	100	100
BSA	> 50,000	19.7 ±3.2	6.7 ±0.8	2.5 ±0.2
	< 1,000	77.1 ±2.9	92.9 ±1.1	97.2 ±0.4

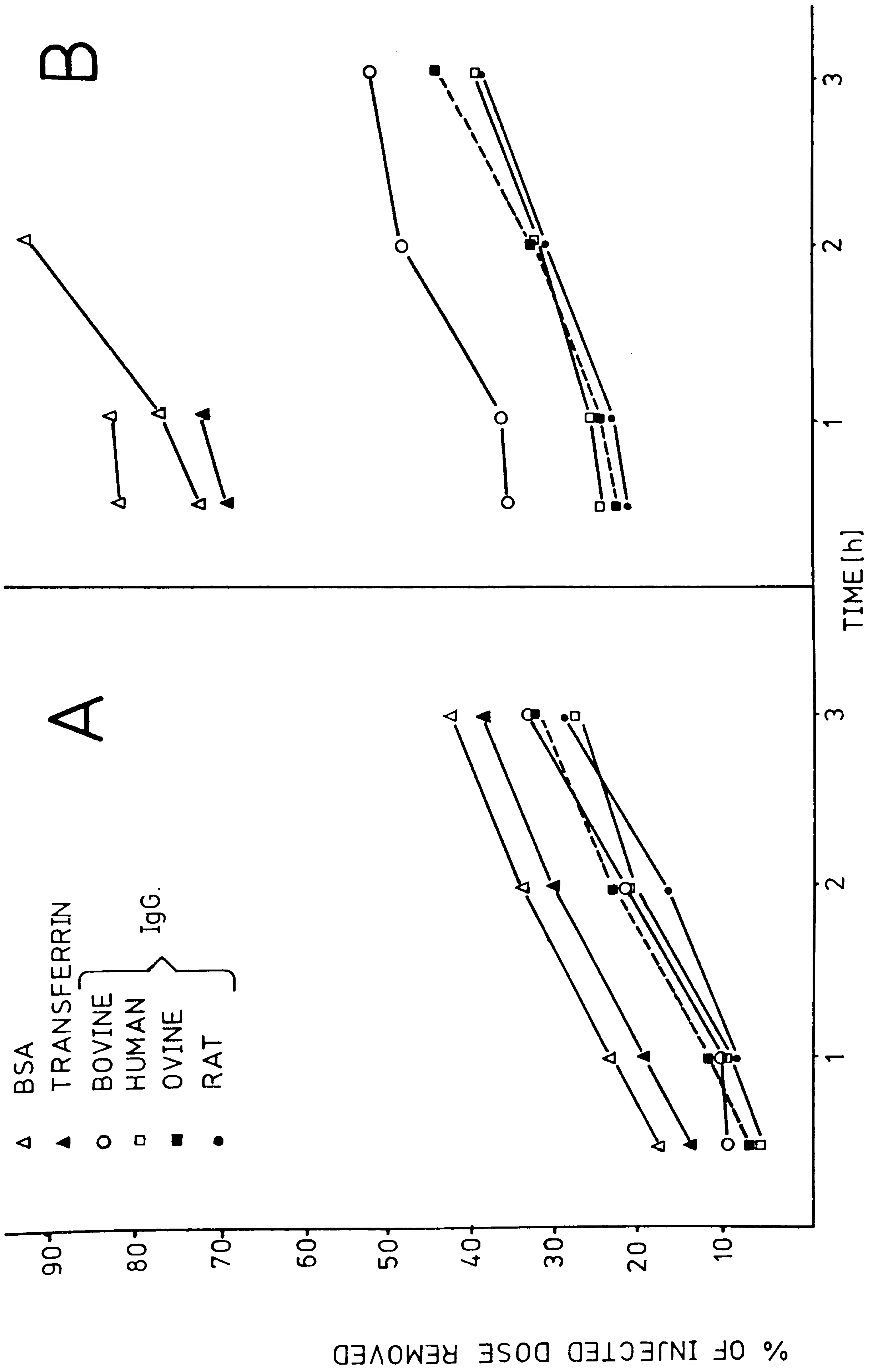
Table 4.4 The relative digestibility of labelled proteins. The amount of non-TCA precipitable radioactivity in gut wash samples, after 2 hr and 4hr incubation is expressed as a percentage of the starting protein-bound activity (mean and S.E. of four samples).

Labelled protein	Percentage of non-TCA precipitable radioactivity after digestion	
	2 hr incubation	4 hr incubation
Bovine IgG	7.1 ±0.3	14.4 ±0.2
Sheep IgG	6.8 ±0.4	11.2 ±0.4
Human IgG	11.2 ±0.1	15.1 ±0.1
Rat IgG	7.8 ±0.3	10.1 ±0.1
Transferrin	33.4 ±0.2	54.9 ±0.5
BSA	6.9 ±0.8	9.9 ±0.7

Table 4.5 Regression analysis of the percentage of the injected samples removed from the vascular space (based on values at 1, 2 and 3 hr).

Sample	No. of animals	Slope (a)	Intercept (b)	Standard Error of regression (y = aX + b)
A. Intact proteins				
Bovine IgG	15	11.5	0.9	±3.5
Sheep IgG	13	7.8	6.1	±3.0
Human IgG	12	8.9	2.1	±3.8
Rat IgG	23	10.1	-1.6	±5.4
Mean		9.6	1.9	
Transferrin	15	8.8	12.8	±3.4
B.S.A.	14	9.8	14.3	±2.9
B. Serum perfusates after ultrafiltration				
Bovine IgG	12	8.2	30.0	±4.0
Sheep IgG	12	9.2	15.8	±3.0
Human IgG	12	7.3	18.8	±2.8
Rat IgG	9	8.1	15.8	±2.4
Mean		8.2	20.1	
B.S.A.	12	11.2	60.0	±4.2
(i)	All regressions are significant at $P < .001$			
(ii)	Differences in slope are non-significant within and between groups A & B			
(iii)	Differences in intercept are significant at .001 level of P between:			
	Rat IgG & Sheep IgG;			
	Rat IgG & B.S.A.;			
	Rat IgG & Transferrin;			
	Intact proteins & perfusates.			

Fig. 4.10 The rates of removal from the vascular compartment of protein and protein fragments, expressed as percentage of the amount injected. A, intact proteins; B, ultrafiltered serum proteins.



transferrin, gave very high initial rates of clearance when injected into the heart. With both of these proteins the radioactivity of the serum was much lower than with the immunoglobulins. Particular difficulty was associated with the perfusate obtained after the intra-intestinal injection of transferrin, since all of the radioactivity in the perfusate was associated with material of less than 1,000 mol. wt. and no concentration effect was obtained with the 50,000 mol. wt. filter. Consequently the radioactivity/ml. remained very low and because of this, radioactivities recorded more than one hour after the intra-cardiac injection of this perfusate were unreliable.

DISCUSSION

Labelled rat IgG leaves the vascular compartment at about 9-10% per hour (Morris and Morris, 1976). The input of rat IgG by enterocytes, after the injection of 1 mg of labelled rat IgG into the proximal small intestine, greatly exceeds this removal rate. Thus intact IgG accumulates in the vascular space. The rates at which bovine, sheep and human IgG leave the vascular space are very similar to each other and to the removal rate of rat IgG, (Fig. 4.10 Table 4.5) and these heterologous IgGs also accumulate in the vascular space after intra-intestinal injection. The amount of intact human IgG transmitted was comparable to that of rat IgG and both of these were significantly greater than the amounts of bovine or sheep IgG transmitted. Proximal enterocytes transmitted intact IgGs preferentially in the order rat, human, sheep and bovine; but the removal of transmitted molecules from the vascular compartment was non-preferential and occurred at about the same rate.

Although considerable intact IgG is transmitted, much more is broken down during passage from the gut lumen through the proximal enterocytes. It has been estimated (Morris and Morris 1977^b) that about 39% of the

labelled rat IgG removed from the intestine by proximal enterocytes is transmitted intact and the rest is broken down into small fragments of less than 1,000 mol. wt. Heterologous IgGs are evidently processed in a similar way: the ultrafiltration studies reported in this chapter (Table 4.3) show that molecules were either transmitted intact or were degraded into fragments smaller than 1,000 mol. wt.

The TCA precipitable radioactivity present in the vascular compartment (Table 4.1) may not have been entirely due to intact molecules. It has been shown that some 33% of IgG fragments up to 1S size may be precipitated by TCA (Morris and Morris 1977a) and that molecular size must be determined by other means. However, an estimate of the amount of intact IgG transmitted, expressed as a percentage of the amount of the dose which had been removed from the intestine, can be obtained from the information in Tables 4.1 and 4.3. Thus for bovine IgG 10.5% of the dose was present in the vascular space (equal to 104.5 μ g of protein); about 57% of this was greater than 100,000 mol. wt. (equal to 59.9 μ g of protein), which is about 10% of the bovine IgG removed from the intestine. Similarly about 13% of the sheep IgG passed through the intestine intact, about 26% of the human IgG and about 35% of the rat IgG. Since a little IgG left the vascular compartment during the two hour experiment, these values are probably under estimates.

All of the human transferrin removed from the intestine was broken down to small fragments (less than 1,000 mol. wt., see Table 4.3), some of which were TCA precipitable. About 20% of the radioactivity in the vascular compartment after the injection of BSA was greater than 50,000 mol. wt.; and density gradient fractionation of serum revealed some radioactive material with a sedimentation coefficient of 4.5S, similar to intact BSA. From the information in Tables 4.1 and 4.3 it can be calculated that 14 μ g of BSA were transmitted intact - about 1.6% of the

BSA removed from the intestine.

Density gradient fractionation of serum after the injection of IgG, revealed that the radioactive material with a molecular weight greater than 100,000 had a sedimentation coefficient of 7S, equal to that of intact IgG.

There appeared to be no significant difference between the amount of radioactive material remaining in the lumen of the proximal small intestine after the injection of rat, human, sheep or bovine IgG (Table 4.1). This is in close agreement with previous studies (Hemmings 1957; Hemmings and Oakley 1957) which demonstrated that heterologous IgGs are taken up in equal quantities by the rabbit yolk-sac splanchnopleur. This evidence suggests that the absorption of IgG from the intestinal lumen is non-selective. However, the amount of intact IgG transported to the serum depends upon its species of origin, and it has been demonstrated that the clearance rates of the various IgGs are very similar. Thus it would appear that selection occurs after entry of the IgGs into the enterocytes but before their appearance in the vascular compartment.

Since the enterocytes removed almost equal amounts of the heterologous and homologous IgGs from the intestinal lumen, the radioactivity which remained in the gut wall after two hours was dependent upon the rate at which the various immunoglobulins were processed by the cells of the proximal small intestine. The results in Table 4.1 show that the immunoglobulins which were least readily transported intact had the most rapid passage through the gut wall. Table 4.1 also shows that as the amount of intact IgG transported increased in the order bovine < sheep < human < rat, so the amount of IgG breakdown products present in the viscera and carcass increased in the reverse order, rat < human < sheep < bovine. The implication of these findings is that as the goodness of fit between the IgG and the receptor decreased, so fewer immunoglobulin molecules were

transported across the cell via the "protected" receptor mediated pathway. The remainder of the IgG which was not protected was digested intracellularly - presumably by the lysosomal system. The IgG breakdown products passed out of the enterocytes much more rapidly than the receptor bound IgG. Consequently, the better the fit between the IgG molecule and the receptor, the slower will be the passage of IgG through the cell because more molecules will be transported via the slow protected pathway. In addition fewer IgG breakdown products will accumulate in the viscera plus carcass.

In accord with the findings of Halliday (1958), the interference studies (Table 4.2 and Fig. 4.9.) clearly demonstrated that the ability of one IgG molecule to interfere with the intact transmission of another IgG molecule is related to their species of origin. The degree to which unlabelled IgGs and proteins could affect the transmission of labelled rat IgG was a reflection of the capacity of the unlabelled molecule to compete with the labelled rat IgG molecule for the Fc receptor. Figure 4.9 shows that BSA and Transferrin had no affinity for the receptor, whereas the affinity of the IgGs increased in the order bovine < sheep < rat < human. This appears to confirm the hypothesis that the amount of IgG transmitted intact is related to its goodness of fit with the receptor.

An alternative explanation of why different amounts of IgGs were transmitted intact by proximal enterocytes could be that, despite their affinity for the receptor, they were digested to differing extents in the gut lumen. The results in Table 4.4 show that this is unlikely since the IgGs rank in the following order with respect to digestibility: rat, sheep, bovine, human, whereas the ranking order for transmission was rat, human, sheep, bovine. Relative digestibility may be a minor factor in determining the number of intact molecules available for transmission, but the degree of affinity for the receptor is much more important.

The relative indigestibility of BSA may explain why a small proportion of the injected dose appeared to be transmitted across the gut intact.

The substances which leave the proximal enterocytes, intact molecules and fragments, pass into the vascular system and eventually reach the tissues, presumably via the capillary beds. Heterologous and homologous IgGs leave the vascular compartment at comparable rates, and if the capillary beds are acting as filtration devices the results in Table 4.5 and Fig. 4.10 show that there is no significant difference in the rates of clearance for molecules in the approximate size range 60,000-158,000 mol. wt. However, small fragments (less than 1,000 mol. wt.) are cleared very rapidly from the vascular compartment.

CHAPTER 5DEGRADATION OF IgG IN THE SMALL INTESTINEINTRODUCTION

The transmission of intact IgG from the suckling rat gut to the circulation is accompanied by a substantial amount of proteolysis (Bangham and Terry 1957; Jones 1972, 1978; Morris and Morris 1976 , 1977a, b, 1978).

The bulk of these breakdown products have been shown to have a molecular size of 2.55-4.55 S when analysed by sucrose density gradient ultracentrifugation (Hemmings 1975b; Morris and Morris 1977a). The studies reported in this chapter attempted to explore the digestion of IgG in the gut wall and gut lumen of both proximal and distal regions of the small intestine. The proteolytic activity of the luminal contents was assayed in rats aged 9, 14 and 19 days in order to examine the distribution of proteolytic enzymes along the length of the small intestine, and to monitor any maturational changes which may occur.

It has been stated (Borthistle 1977) that the receptors present on the surface of proximal enterocytes are sensitive to treatment with trypsin. Experiments were performed to examine the effect of trypsin on the ability of the proximal small intestine to transport intact IgG to the circulation *in vivo*.

The present study also attempted to assess the influence of trypsin inhibitor on the transfer of intact IgG across the gut.

MATERIALS AND METHODSGel filtration

Gel filtration was performed on gut washes and gut wall homogenates prepared from the ligated proximal and distal regions of the small intestine, 15 minutes - 3 hours after the intraluminal injection of labelled rat IgG.

The gut lumen was washed out with 1.0 ml of ice cold 0.2M Sucrose in 0.1M MES buffer at pH 6.2. The wash was centrifuged at 6,500 g for five minutes to remove any particulate material. The gut wall was homogenized in 5.0 ml of 0.2M Sucrose in 0.1M MES buffer in a Potter-Elvehjem homogenizer. The resulting homogenate was sonicated to effect complete cellular disruption, and then centrifuged at 1,000 g for 10 minutes to pellet any large debris. The supernatant was subsequently concentrated to a volume of approximately 1.0 ml by rotary evaporation.

0.5 ml samples of the gut washes and gut wall extracts were applied to a 2.6 cm x 40 cm column of Sephadex G.100 at room temperature, using 0.1M MES buffer at pH 6.2 as eluent. The column was first calibrated by running 10 mg samples of the following molecular weight markers:- IgG (150,000 mol. wt.), haemoglobin (64,000 mol. wt.), pepsin (36,000 mol. wt.) and myoglobin (17,800 mol. wt.).

Constant volume fractions were collected by means of an LKB Ultrarac fraction collection system at a flow rate of 30 ml per hour. These fractions were assayed for protein and radioactivity.

Analysis of the influence of ligation on luminal proteolytic activity

This assay was intended to assess the influence of ligation on the luminal proteolytic activity of the proximal small intestine. The animals were operated on and the proximal intestine ligated as already described. At various times after the injection of 0.2 ml of labelled rat IgG, the animals were killed and the ligated section of gut was washed out with 5 ml of ice cold 0.20M sucrose in 0.1M phosphate buffer at pH 6.0. The gut wash was centrifuged at 6,500 g for 15 minutes in order to remove any particulate material. Enzyme assays were performed on aliquots (50 μ l) of the gut wash which were added to 4.95 ml of 0.1M phosphate buffer at pH 7.0, containing 25 mg of Azocoll (Calbiochem). [Azocoll is an insoluble,

powdered cowhide to which a bright red dye is attached. The cowhide contains the wide assortment of peptide linkages characteristic of all proteins and so is a good general proteolytic substrate. When a proteolytic enzyme breaks one of these linkages, the bound dye is released into the suspending medium. The rate at which the dye is released is used to measure the proteolytic activity in a solution.] The digestive action of the proteases in the gut wash was terminated by filtering off the undigested Azocoll with Whatman No. 1 filter paper. The soluble products of Azocoll digestion were assayed by measuring the absorbance at 520 nm.

IgG transport from a trypsin treated proximal intestine

The effect of a prior treatment of the proximal small intestine with trypsin on the ability of this region of the gut to transport intact IgG was examined.

0.2 ml of trypsin solutions, ranging in concentration from 1.0 to 10.0 mg/ml, were injected into the ligated proximal small intestine using the operative procedure previously described. One hour later, 0.2 ml of labelled rat IgG containing trypsin inhibitor (from Soybean; Sigma Chemical Co.) was also injected into the ligated segment of intestine. The trypsin inhibitor was present at a concentration equal to that of the trypsin solution injected into the gut one hour previously, and was included to prevent excess luminal digestion of the IgG by the introduced trypsin.

The animals were killed two hours after the injection of the labelled IgG into the gut, and the serum and intestine were assayed for radioactivity.

Control animals were operated on in an identical fashion, except that in place of trypsin 0.2 ml of 0.1 MES buffer at pH 6.2 was injected into the duodenum.

The effect of trypsin inhibitor on IgG transport

Studies were performed to examine the effect of trypsin inhibitor on the transport of intact IgG across the proximal small intestine.

Using the standard operative procedure, a 0.2 ml dose containing labelled rat IgG (1 mg) and 1.0 mg of trypsin inhibitor, was injected into the lumen of the ligated proximal small intestine. The distribution of radioactivity in the intestines, serum and carcass was subsequently determined, as described previously.

The procedure was repeated using a 0.2 ml dose containing labelled rat IgG (0.25 mg) and 0.2 mg of trypsin inhibitor.

Measurement of luminal proteolytic activity

The small intestine was dissected from neonatal rats aged 9, 14 and 19 days and divided into six portions of equal length. Each segment was washed out with 1 ml of ice cold 0.2M Sucrose in 0.1M MES buffer at pH 6.2. The washes were centrifuged at 6,500 g for 5 minutes to remove any particulate material, and the supernatants were stored at -14°C for estimations of proteolytic activity.

The luminal proteolytic activity was determined by incubating 0.2 ml of the gut wash with 0.25 ml of 8% (W/V) haemoglobin solution and 0.55 ml of 0.1M TRIS/HCl buffer (pH 8.0) at 37°C for 30 minutes, in a shaking water bath. The reaction was terminated by the addition of 5 ml of 3% TCA. The precipitated undigested protein was spun down at 6,500 g for 5 minutes. The degree of proteolysis was determined by measuring the absorption of the liberated amino acids in the supernatant at 280 nm.

RESULTS

Gel filtration

Labelled rat IgG was injected into either the isolated proximal or the isolated distal region of the small intestine. After varying time periods of up to three hours, the luminal contents were washed out, centrifuged and the supernatant subjected to gel filtration on a Sephadex G 100 column. Extracts prepared from gut wall homogenates were also subjected to gel filtration. The profiles obtained from the proximal and distal gut walls and washes are shown in Fig. 5.1 and Fig. 5.2 respectively.

The results in Fig. 5.1 show that there was some degradation of IgG in the lumen of the proximal small intestine. After two hours there is a well defined peak of breakdown products with a molecular weight of about 60,000. However the corresponding profile of the gut wall extract showed that the enterocytes contained less of these breakdown products, and appeared to contain mostly intact IgG.

The profiles obtained from digestion of IgG in the ileal lumen were found to be similar to those from the proximal region of the small intestine, with the major degradation product having a molecular weight of 60,000. However the profile for the distal wall extract was different from that of the proximal wall. The radioactivity in the ileal enterocytes was mostly in the form of large breakdown products.

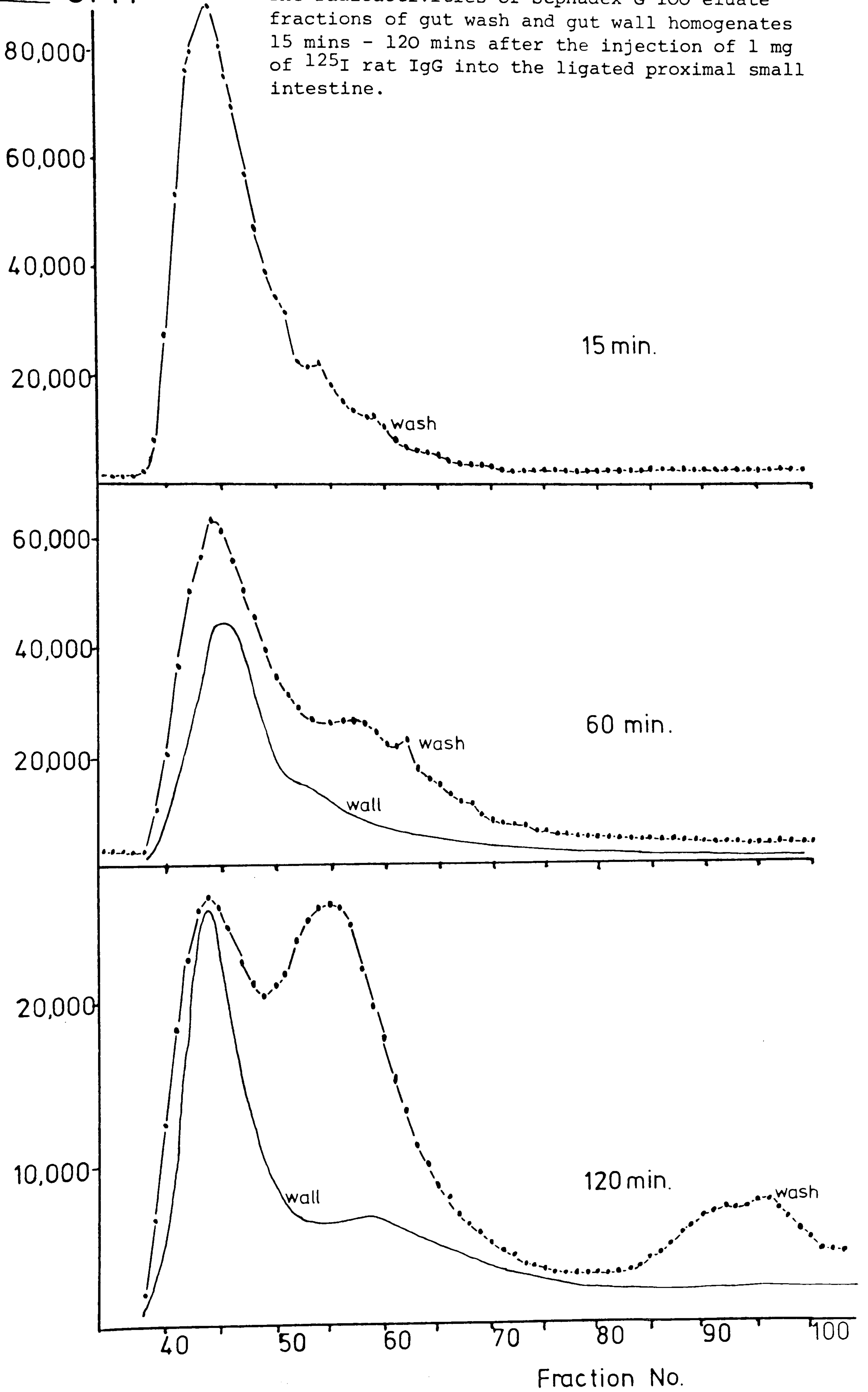
Fig. 5.3 shows the molecular weight calibration curve used for the sephadex G 100 column.

Analysis of the influence of ligation on luminal proteolytic activity

During the course of experiments, the isolation of the proximal small intestine by ligation may have restricted the flow of pancreatic and other enzymes into the gut lumen. Luminal washings were collected from ligated guts at various time intervals up to two hours after the

Fig. 5.1 CPM

The radioactivities of Sephadex G 100 eluate fractions of gut wash and gut wall homogenates 15 mins - 120 mins after the injection of 1 mg of ^{125}I rat IgG into the ligated proximal small intestine.



The radioactivities of Sephadex G 100 eluate fractions of gut wash and gut wall homogenates 15 mins - 120 mins after the injection of 1 mg of ^{125}I rat IgG into the ligated distal small intestine.

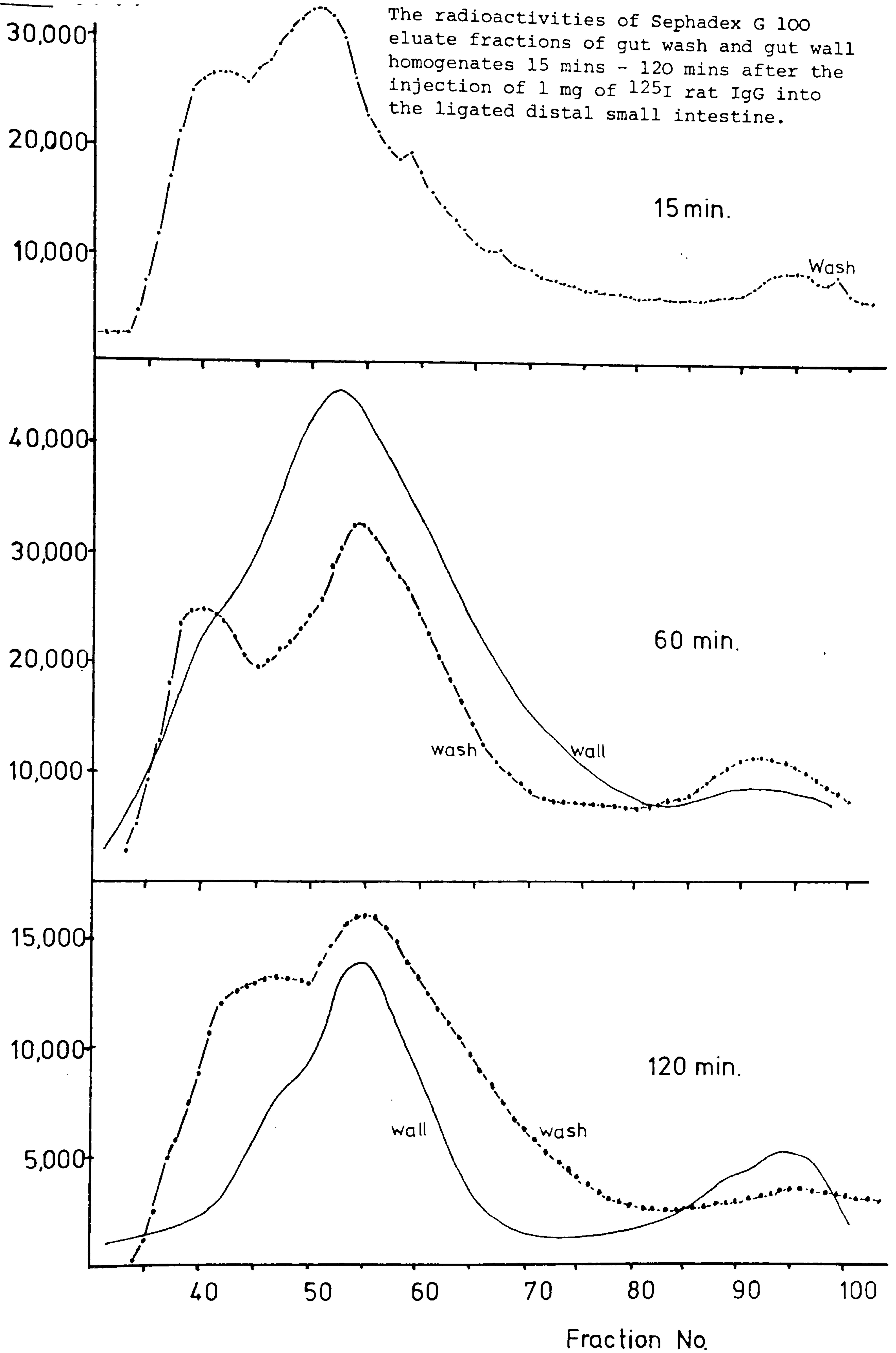
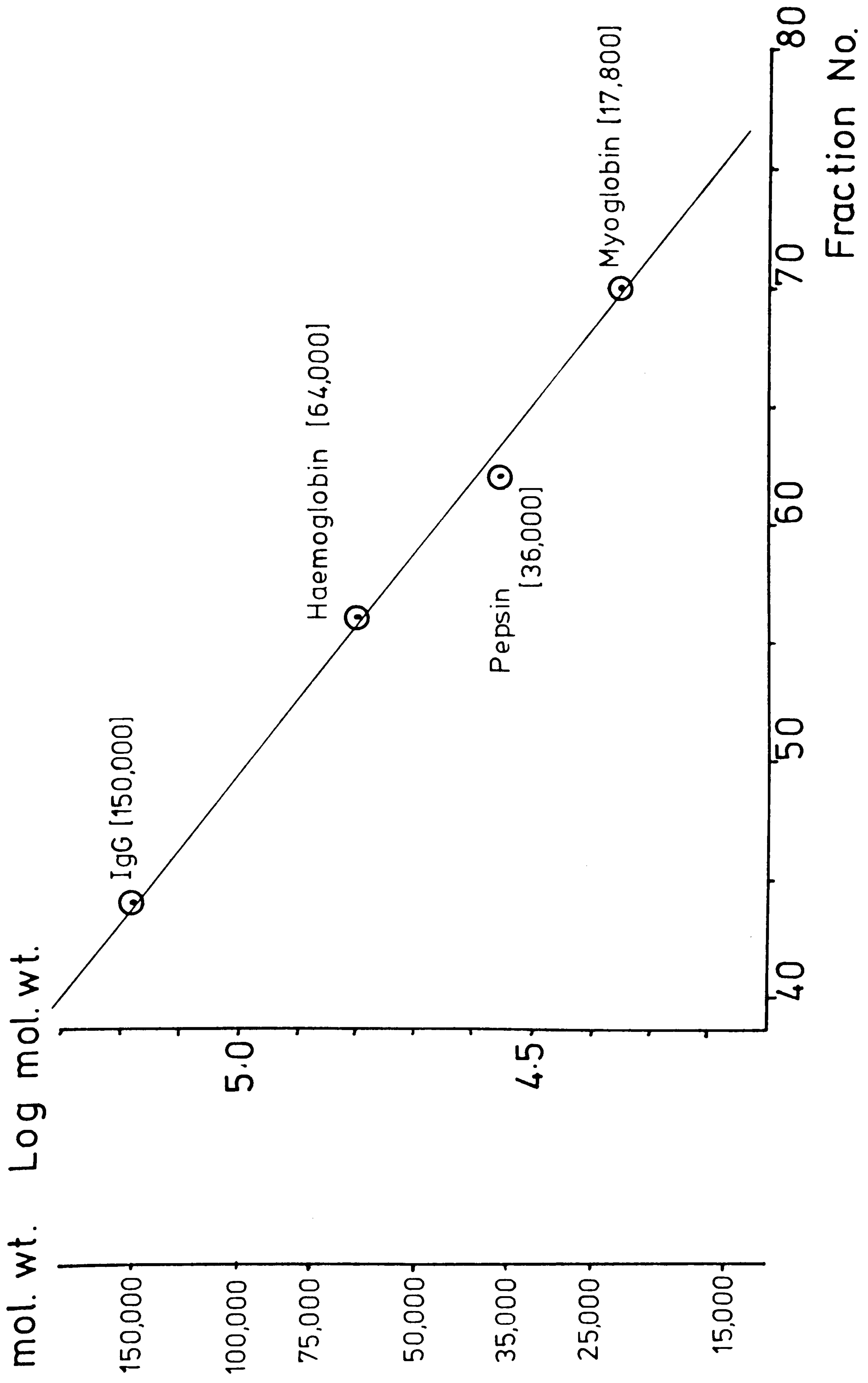


Fig. 5.3 Molecularweight calibration curve for Sephadex G 100 column.



intraluminal injection of IgG, and the proteolytic activity of the contents were assayed using Azocoll. The results in table 5.1 represent the proteolytic activity of the luminal contents at various times after ligation, assuming the activity at time zero was equal to 1.0. The results demonstrated that up to two hours after ligation of the proximal small intestine, there is no reduction in the luminal proteolytic activity.

Table 5.1 Estimation of luminal proteolytic activity using Azocoll. The results are given as the mean (\pm S.E.M.) assuming the proteolytic activity at time zero is equal to unity. The number of samples are shown in parentheses. The time represents the period between intraduodenal injection of IgG, and luminal washing.

TIME (min)	RELATIVE PROTEOLYTIC ACTIVITY		
0	1.00	± 0.09	(4)
15	1.21	± 0.40	(5)
60	1.87	± 0.77	(5)
120	1.43	± 0.35	(4)

IgG transport from a trypsin treated proximal small intestine

It has been claimed that the receptors for monomeric IgG on the luminal surface of enterocytes in the proximal small intestine are sensitive to treatment with trypsin (Borthistle 1977). In order to examine this contention, a series of tests were performed to quantify the effect that a prior exposure to trypsin would have on the ability of the proximal region of the gut to transport intact IgG to the circulation.

Table 5.2 gives the mean values (\pm S.E.M.) for the amount of T.C.A. precipitable radioactive material present in the vascular compartment two

hours after the injection of labelled rat IgG, into trypsin treated proximal small intestines.

There appeared to be no significant reduction in the concentration of T.C.A. precipitable radioactive material in the vascular compartment of trypsin treated animals compared with the controls.

Table 5.2 T.C.A.-precipitable radioactivity in the vascular compartment, two hours after injection of labelled IgG into a trypsin treated gut. The values represent the means (\pm S.E.M.), and the number of animals is shown in parentheses.

TRYPSIN CONCENTRATION (mg/ml)		T.C.A. PRECIPITABLE RADIOACTIVITY IN THE VASCULAR COMPARTMENT (μ g)		
0.1	EXPERIMENTAL	156.0	\pm 11.5	(8)
	CONTROL	152.7	\pm 18.5	(4)
1.0	EXPERIMENTAL	154.5	\pm 8.6	(5)
	CONTROL	143.3	\pm 21.5	(4)
10.0	EXPERIMENTAL	244.6	\pm 19.2	(9)
	CONTROL	234.9	\pm 25.2	(4)

The effect of trypsin inhibitor on IgG transport

These studies were performed to examine the potential significance of trypsin inhibitor on the selective transmission of rat IgG in the neonatal rat gut.

1 mg of labelled rat IgG and 1 mg of trypsin inhibitor in a 0.2 ml dose were injected into the ligated proximal small intestine, and the transmission of IgG was monitored after a two hour incubation period.

At this concentration, the amount of IgG injected was sufficient to saturate the uptake mechanism (Chapter 6 Table 6.1). In a second experiment, the concentration of the IgG injected was reduced to 1.25 mg/ml which was below the saturation level. The results of both experiments are summarized in Table 5.3.

It was evident that injection of trypsin inhibitor with the labelled rat IgG, did not increase the level of T.C.A. precipitable radioactivity present in the vascular compartment after a two hour incubation period.

Table 5.3 The transport of labelled rat IgG from the proximal small intestine after two hours in the presence of trypsin inhibitor. The values are expressed in μg of protein and represent the mean \pm S.E.M. The number of samples is shown in parentheses.

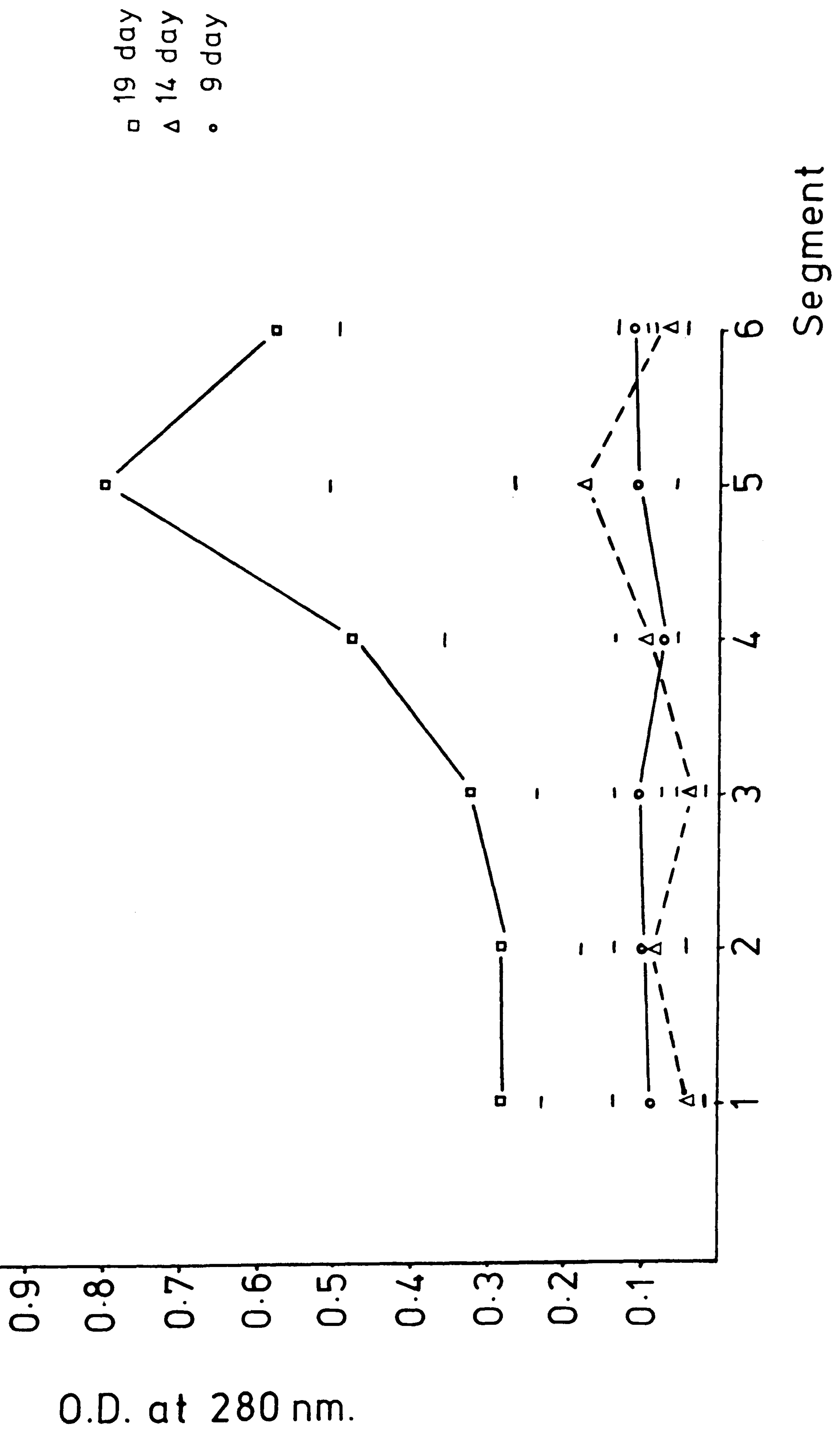
Dose of IgG injected (μg)	Dose of Trypsin Inhibitor Injected (μg)	Radioactivity Remaining in the gut	Radioactivity Removed		
			Vascular Compartment		Viscera & Carcass
			T.C.A.- precipitable	T.C.A.- soluble	
1000 (6)	1000	729.2 \pm 9.0	172.8 \pm 5.1	5.2 \pm 1.1	92.6 \pm 11.4
1000 (16)	0	552.3 \pm 16.2	188.4 \pm 7.8	21.2 \pm 3.2	238.1 \pm 12.6
250 (5)	250	150.2 \pm 2.2	91.4 \pm 8.5	1.1 \pm 0.2	7.4 \pm 4.3
250 (10)	0	129.7 \pm 6.8	109.7 \pm 11.4	1.8 \pm 0.2	8.8 \pm 5.3

It was of interest to note from Table 5.3 that at the higher IgG concentration, considerably more radioactivity was removed from the gut in the absence of trypsin inhibitor. The results indicate that the extra radioactivity removed accumulated in the viscera and carcass.

The measurement of luminal proteolytic activity

The results presented in Fig 5.4 show the distribution of luminal proteases along the length of the small intestine in 9, 14 and 19 day old

Fig. 5.4 The distribution of luminal proteolytic activity in intestinal segments 1 (pyloric end) to 6 (ileo-caecal end) of the small intestine, measured at pH 6.2. Each point represents the mean \pm S.E.M. from four animals.



rats. It was observed that there was no significant difference in the levels of luminal proteases at the ages of 9 and 14 days, and that this activity remained at a constant level along the length of the small intestine. However there was an increase in protease levels at 19 days of age, and this activity was highest in the distal region of the small intestine.

DISCUSSION

It has previously been assumed that the immaturity of the luminal digestive system in the suckling rat, as described by Hill (1956), ensured the non-digestion of maternal immunoglobulins in the milk as they traversed the stomach and small intestine. Jones (1972) however, showed pepsin and trypsin to be present in the gut, and that luminal digestion of IgG was substantial. In these experiments, Jones allowed labelled IgG to travel the entire length of the small intestine, which was subsequently flushed out and the luminal contents examined by gel filtration. No account was taken of the possibility that the luminal proteolytic activity may change along the length of the gut. Furthermore, the IgG fed to the young rats was buffered at pH 8, which is close to the optimum value for trypsin activity. The normal pH of the proximal small intestine is close to pH 6 (Rodewald 1976c), and at this low pH there is unlikely to be a great deal of tryptic activity. These factors may have led to overestimations of the level of proteolytic activity in the duodenum and jejunum.

In a subsequent study, Jones (1978) demonstrated marked regional differences in luminal proteolysis along the small intestine. The results summarized in Fig. 5.1 and 5.2 confirm these findings. The major product of the luminal digestion of IgG in both proximal and distal regions of the small intestine, appeared to have a molecular weight of

approximately 60,000.

Fifteen minutes after injection, more than half of the radioactivity present in the lumen of the distal small intestine was associated with IgG breakdown products (Fig. 5.2). However, in the proximal region of the gut (Fig. 5.1) significant quantities of these high molecular weight IgG breakdown products did not become apparent until two hours after injection. Thus the proteolytic activity of the proximal lumen is much lower than that of the distal lumen, even though assays on the level of proteases in the lumen of the small intestine of 14 day old rats revealed no differences between the proximal and distal regions (Fig. 5.4).

Rodewald (1976c) found the luminal pH of the proximal small intestine to be between 6.2 and 6.3. This value increased in the distal regions of the small intestine, and reached pH 6.9-7.4 in the ileum. Thus although the level of luminal proteases did not increase along the length of the small intestine in the 14 day old rat (Fig. 5.4), the proteolytic activity would be greater in the distal lumen since the pH of this region is closer to pH 8, which is the optimum for luminal proteolytic enzymes (Jones 1972).

It is interesting to note that two hours after the injection of labelled IgG into the proximal small intestine, the gut wall appeared to contain mainly intact IgG, even though there were significant quantities of IgG breakdown products in the lumen of the gut. This suggests that the proximal enterocytes were transporting mainly intact IgG to the circulation at this time.

In contrast, the cells of the ileum contained mostly breakdown products, which is a reflection of the digestive role of this region of the small intestine.

It was necessary to examine the effect of ligation of a section of

the proximal small intestine, on the luminal proteolytic activity. This was to demonstrate that the results obtained from IgG transmission studies (Chapters 4 and 6) and that the gel filtration analysis of luminal washes were not artifacts produced by this operative procedure. The data from enzyme assays, using Azocoll as a general proteolytic substrate, demonstrated that up to two hours after ligation there was no reduction in the digestive capacity of the luminal contents (Table 5.1).

The existence of trypsin sensitive Fc-receptors on the plasma membrane of macrophages is well documented (Silverstein 1977). These receptors selectively bind to certain monomeric IgG subclasses when the antibody molecule is not in the form of an antigen-antibody complex. Macrophages and polymorphonuclear leucocytes also express a protease resistant Fc-receptor which mediates the efficient binding and ingestion of IgG-antigen complexes.

Borthistle *et al* (1977) examined the binding of labelled immunoglobulins to the cells of an isolated loop of jejunum. To prevent intracellular absorption of proteins during the incubation period, the solutions injected into the jejunal loop contained 10 mM sodium fluoride. Borthistle claimed that the receptors on the luminal surface of fluoride treated jejunal enterocytes were sensitive to trypsin treatment, and a mechanism was suggested by which IgG binding and transport could be terminated under physiological conditions. This involved the destruction of Fc-receptors at 21 days of age by the increase in pancreatic secretion which occurs at this time.

Examination of the binding of ^{125}I rabbit IgG to formalin fixed rabbit yolk-sac membrane by Hillman *et al* (1977), also demonstrated the sensitivity of the Fc-receptor to digestion by proteolytic enzymes. He showed that the IgG binding capacity of the yolk-sac membrane was reduced by treatment with papain and trypsin. Wild (1979) examined the same

phenomenon by means of an erythrocyte-antibody rosetting technique. In this assay, sheep red blood cells (SRBC) were sensitised with sub-agglutinating amounts of rabbit anti-SRBC IgG and allowed to react with isolated rabbit yolk-sac endodermal cells. Rosetting cells were observed by high power phase microscopy and their frequency assessed. Rosette formation was greatly reduced by incubating the isolated endodermal cells in a medium containing 0.05% trypsin.

From these *in vitro* binding assays it would seem clear that the IgG Fc-receptor is indeed trypsin sensitive. However, the results obtained in the present study using *in vivo* techniques demonstrated that there was no significant reduction in the transmission of intact IgG to the circulation after treatment of the proximal small intestine with trypsin (Table 5.2). Consequently using this assay technique, the Fc receptor does not appear to be trypsin sensitive.

The physiological importance of colostral trypsin inhibitor in the transmission of passive immunity from mother to neonate is well established in those animals which absorb colostral proteins non-selectively in the first few hours after birth, such as pigs, cows, goats, horses and donkeys (Bainter 1973, 1976; Laskowski 1951, 1958; Laskowski, Kassell and Hagerty 1957; Weström *et al* 1979). The function of these inhibitors is to prevent the digestion of proteins destined for transmission to the circulation. In contrast, the transmission of protein across the suckling rat gut is a selective process, and it has been shown that rat colostrum contains very low levels of trypsin inhibitor (Bainter 1973; Carlsson, Karlsson and Weström 1975).

Recently, Jones (1980) observed that soya-bean trypsin inhibitor reduced luminal digestion of human IgG injected into the ileum, but did not increase the transmission of intact IgG by this region of the gut. Thus the inability of the ileum to transmit intact IgG to the circulation

is not due to the high proteolytic activity in this region, but to the absence of an IgG transport mechanism.

The data presented in Table 5.3 demonstrates that trypsin inhibitor did not increase the amount of intact IgG transmitted to the circulation by the proximal small intestine, neither at IgG concentrations sufficient to saturate the uptake mechanism (see Chapter 6) nor at concentrations well below this level. Thus the degree of luminal digestion in the proximal small intestine is low enough not to significantly affect the transmission of immunoglobulins from mother to neonate.

The results summarized in Fig. 5.4 show the maturational changes in the levels of luminal proteases along the length of the small intestine. Between 9 and 14 days of age, the level remains fairly constant from the duodenum to the caecum. By 19 days of age, the gut begins to develop a more active extracellular digestive mechanism which is characteristic of the adult gut. It is interesting to note that the highest levels of proteases were found in the ileum, even though the duodenum is the point of entry into the gut for pancreatic enzymes. This distribution of proteolytic enzymes is similar to that reported by Pelot and Grossman (1962).

CHAPTER 6

KINETIC AND SATURATION STUDIES ON THE TRANSMISSION OF RAT IgG ACROSS THE
PROXIMAL SMALL INTESTINE

INTRODUCTION

The various models which have been proposed to explain the trans-cellular transport of IgG, make certain predictions concerning the fate of receptor bound and non receptor bound immunoglobulin molecules within the cell. In order to obtain further insights into the mechanisms involved in the proximal small intestine of the suckling rat, a study was made of the rate of transmission of an intra-luminally administered dose of labelled IgG, and the subsequent appearance of intact IgG and IgG breakdown products in the viscera and carcass, and the vascular compartment.

The saturability of the IgG transport system was quantified, and the way in which the proximal small intestine dealt with doses both well above and well below the saturation level, was examined.

The implications of these saturation and kinetic studies are discussed with reference to the mechanisms of IgG transmission.

METHODS

Saturation studies

In order to examine the saturability of the IgG uptake mechanism, groups of 15-16 day old animals were operated on as described in Materials and Methods (Chapter 2). 0.2 ml doses of labelled rat IgG, at concentrations ranging from 0.50 - 10.00 mg/ml, were injected into the ligated proximal small intestine. Two hours after injection the animals were killed and a blood sample was collected from the heart. The transport of radioactive material to the serum and the viscera plus carcass was monitored over this two hour period, as outlined previously.

IgG uptake kinetics

A series of experiments was designed to examine the rate of transmission of an intraluminally administered 0.2 ml dose of rat IgG, to the vascular compartment and the viscera plus carcass. Two studies were performed, one at a dose concentration sufficient to saturate the intact IgG transport mechanism (5.00 mg/ml), and a second at a lower concentration (1.25 mg/ml). The transport of radioactive material from the gut to the viscera and carcass and the vascular compartment, was assayed at time intervals ranging from 15 minutes to six hours after the intraluminal injection of IgG.

RESULTS

Saturation studies

Labelled rat IgG (0.2 ml), at concentrations ranging from 0.5 to 10.0 mg/ml, was injected into the ligated proximal small intestine of 15-16 day old rats. Two hours after injection the animals were killed and the transport of radioactive material to the vascular compartment and the viscera plus carcass was determined. The results of these studies are shown in Table 6.1 and Fig. 6.1 and are expressed in μg equivalents of IgG.

From Fig. 6.1 it can be seen that the amount of radioactivity removed from the proximal small intestine is linearly related to the μg of labelled IgG present in the 0.2 ml dose. The straight line relating these two variables has a correlation coefficient of 0.997, and a slope of 0.40. Thus about 40% of the injected dose is removed from the gut after two hours, irrespective of the dose concentration within the 0.5 - 10.0 mg/ml range.

TABLE 6.1 The effect of changing the concentration of the 0.2 ml dose of rat IgG injected into the proximal small intestine, on the radioactivity present in the vascular compartment and the viscera and carcass, two hours after intraluminal injection. The values are given in μg equivalents of protein, and represent the mean \pm S.E.M. The number of animals is shown in parentheses.

DOSE CONCENTRATION (mg/ml)	DOSE (μg)	RADIOACTIVITY REMOVED FROM THE GUT	RADIOACTIVITY IN THE VASCULAR COMPARTMENT		RADIOACTIVITY IN THE VISCERA AND CARCASS
			TCA precipitable	TCA soluble	
10.00 (5)	2000	795.5 \pm 15.6	200.3 \pm 14.9	17.4 \pm 1.6	577.8 \pm 29.2
5.00 (16)	1000	447.7 \pm 16.2	188.4 \pm 7.8	21.2 \pm 3.2	238.1 \pm 12.6
3.75 (5)	750	299.2 \pm 10.2	173.6 \pm 13.0	10.8 \pm 0.4	114.8 \pm 4.6
2.50 (5)	500	240.4 \pm 10.9	167.3 \pm 4.2	4.6 \pm 0.3	68.5 \pm 11.1
1.25 (5)	250	104.4 \pm 2.1	79.9 \pm 5.0	2.1 \pm 0.3	22.4 \pm 3.7
0.50 (5)	100	49.6 \pm 2.5	45.5 \pm 1.6	0.6 \pm 0.1	3.5 \pm 4.0

Fig. 6.1

The effect of changing the concentration of the 0.2 ml dose of rat IgG injected into the proximal small intestine on the radioactivity removed from the gut, and on the radioactivity present in the vascular compartment and the viscera and carcass after two hours.

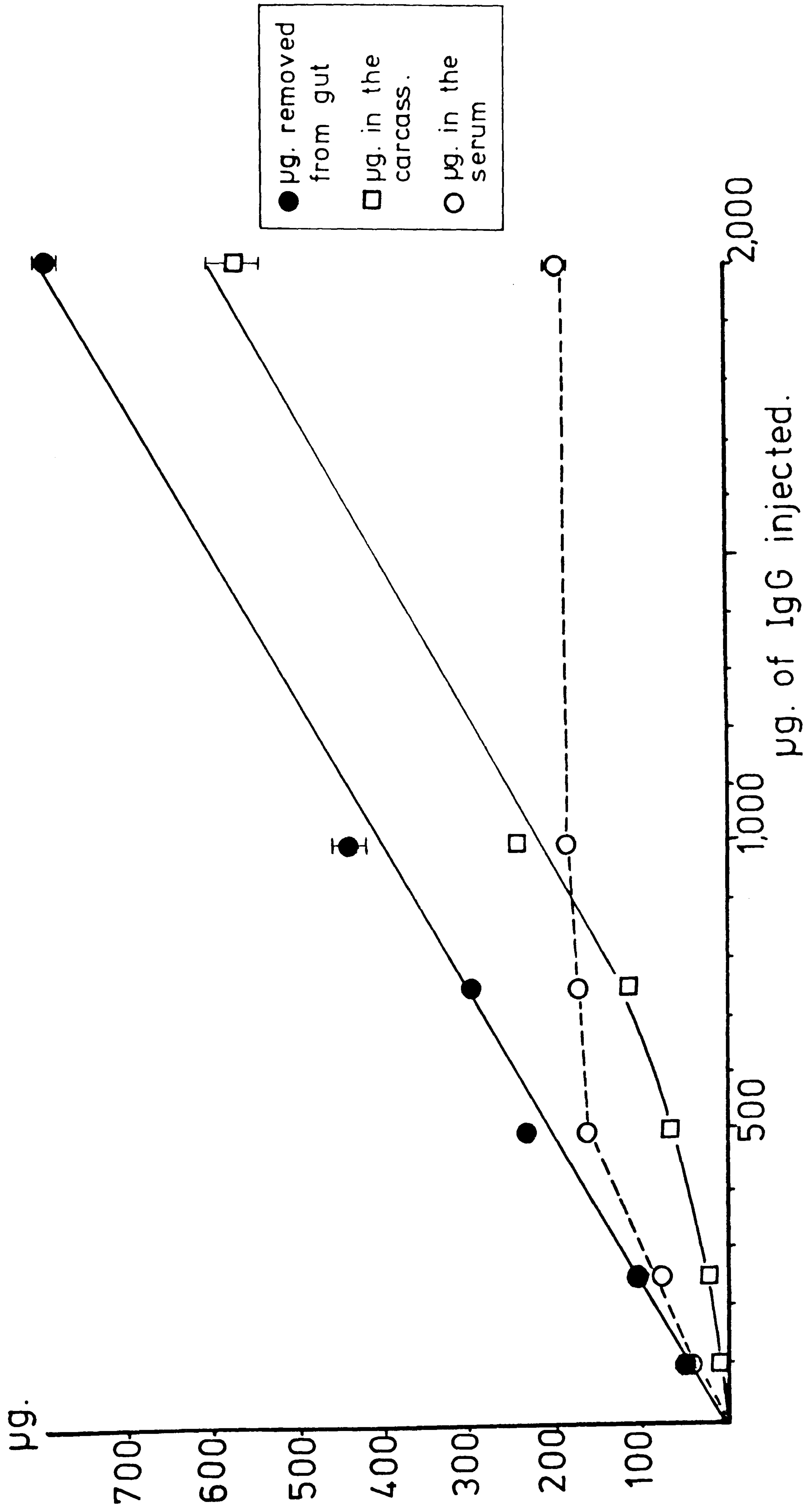


Figure 6.1 clearly shows that the transmission of intact IgG to the vascular compartment is a saturable process, and a dose of between 500 and 750 μ g of IgG in 0.2 ml is sufficient to achieve saturation. Increasing the concentration of the dose beyond this level did not significantly increase the amount of intact IgG transported to the serum. There was, however, a marked increase in the radioactivity associated with the viscera and carcass. Table 6.2 shows the relationship between the concentration of the intraluminally injected dose of IgG, and the percentage of the dose removed from the gut after two hours. The results confirmed that although the dose concentration was increased from 0.50 to 10.0 mg/ml, the percentage of the dose removed remained fairly constant at about 40%.

Table 6.2 also shows that as the dose concentration was increased, so the proportion of the radioactivity removed from the gut, which subsequently appeared as TCA precipitable material in the vascular compartment, decreased. Conversely the proportion of the radioactivity removed which subsequently appeared in the viscera and carcass increased.

TABLE 6.2 The effect of changing the concentration of the 0.2 ml dose of labelled rat IgG injected into the proximal small intestine, on the transmission of radioactive material to the vascular compartment and the viscera plus carcass, after two hours.

DOSE CONCENTRATION (mg/ml)	DOSE (μ g)	% of DOSE REMOVED FROM THE GUT	% OF REMOVED DOSE	
			PRESENT AS TCA PRECIPITABLE RADIOACTIVITY IN THE VASCULAR COMPARTMENT	PRESENT IN THE VISCERA AND CARCASS
10.00	2000	39.8	25.2	72.6
5.00	1000	44.8	42.1	53.2
3.75	750	39.9	58.0	38.4
2.50	500	48.1	69.6	28.5
1.25	250	41.8	76.5	21.5
0.50	100	49.5	91.9	7.1

FIG. 6.2

The relationship between the concentration of the 0.2 ml dose of rat IgG injected into the proximal small intestine and the percentage of the dose removed from the gut after two hours which appeared as TCA-precipitable radioactivity in the vascular compartment (□) and as IgG breakdown products in the viscera and carcass (○).

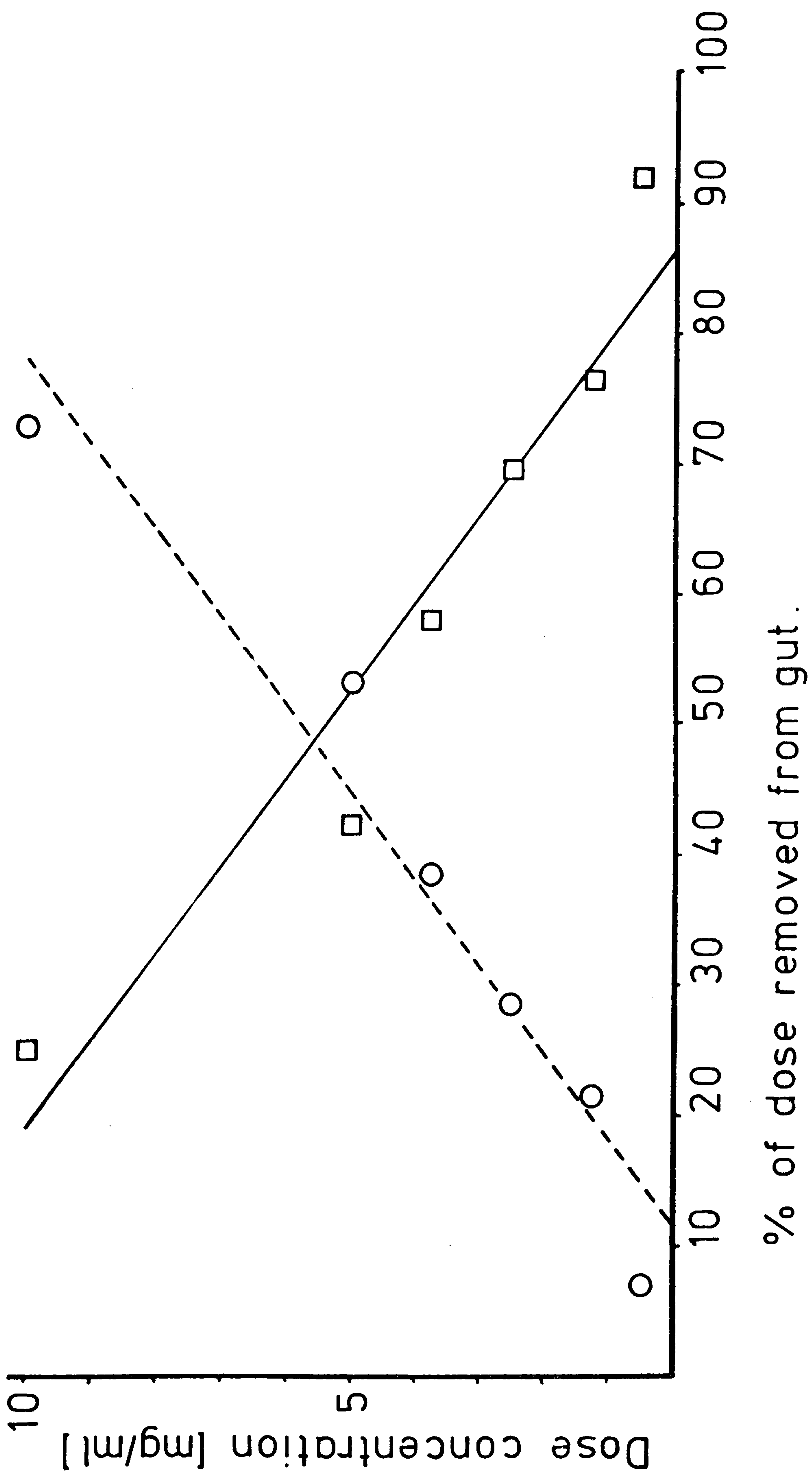


Figure 6.2 shows the relationship between the concentration of the dose injected and the percentage of the dose removed which appeared as TCA-precipitable radioactivity in the vascular compartment and as IgG breakdown products in the viscera and carcass. The equations of these straight lines are as follows:

1) $y = -6.7x + 86.4$ (correlation coefficient = 0.957)

where y = dose concentration (in mg/ml)

and x = % of the dose removed present as TCA-precipitable radioactivity in the vascular compartment.

2) $y = 6.6x + 11.6$ (correlation coefficient = 0.969)

where y = dose concentration (in mg/ml)

and x = % of the dose removed present in the viscera and carcass.

Kinetic studies

The pattern of transmission of radioactive material from the ligated proximal small intestine was examined at varying time intervals (15 min. to 6 hrs.) after the intraluminal injection of ^{125}I labelled rat IgG. Table 6.3 and Fig. 6.3 show the levels of radioactive material (represented as μg equivalents of protein) present in the intestine, vascular compartment and the viscera plus carcass, after the injection of a 1000 μg dose of IgG in 0.2 ml.

The results show that initially there is a slow accumulation of TCA precipitable radioactivity in the vascular compartment. The rate of accumulation gradually increases, and reaches a maximum value of 143.3 μg of IgG per hour between one and two hours after injection. This rate declines thereafter, and the total amount of TCA precipitable labelled IgG present in the vascular compartment attains a plateau of about 350 μg , three and a half hours after injection. This value remains

TABLE 6.3

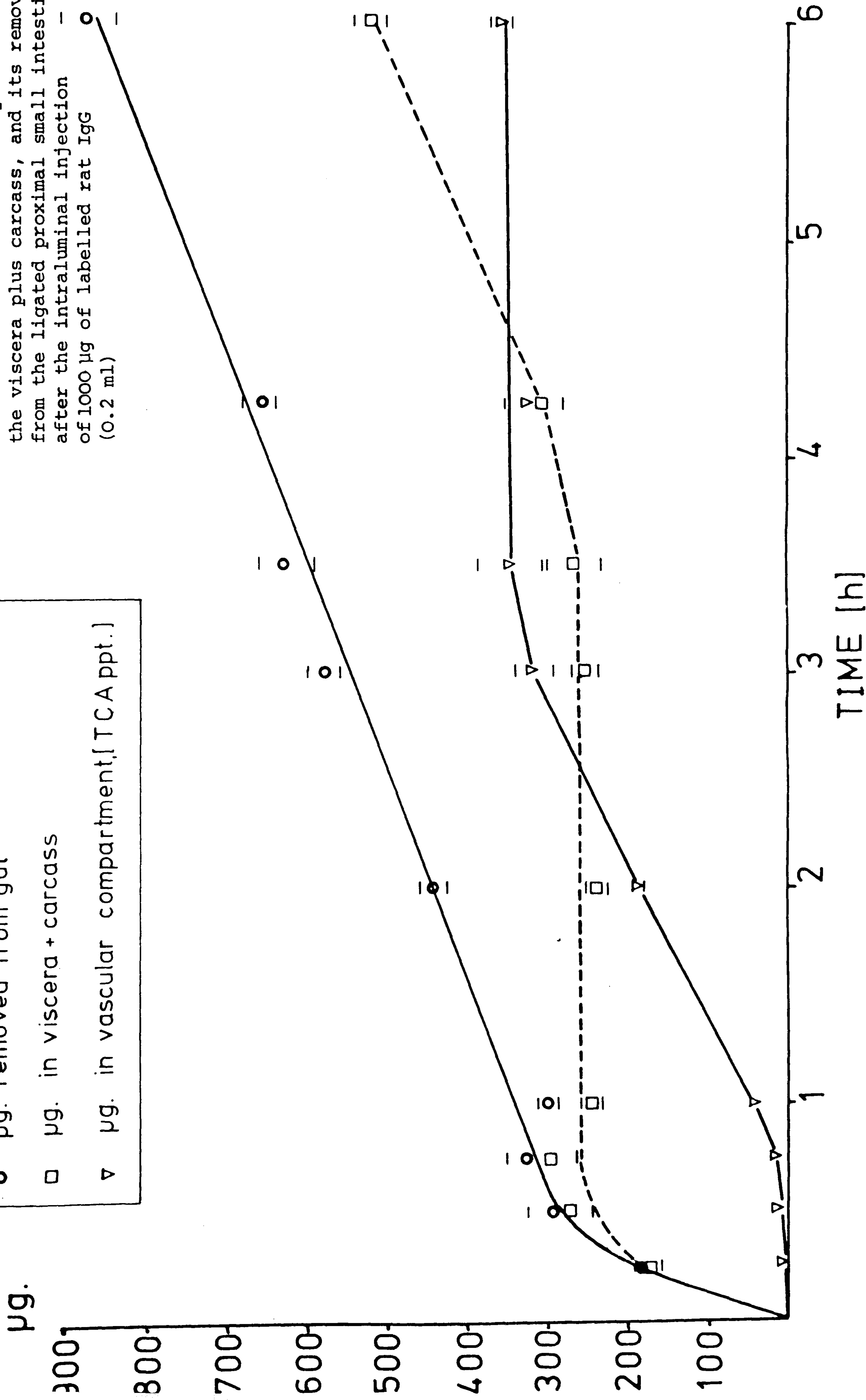
The distribution of radioactive material with time in the intestine, vascular compartment and carcass plus viscera after the intraluminal injection of 1000 μ g of labelled rat IgG into the ligated proximal small intestine. The results, expressed as μ g equivalents of protein, are given as mean values \pm S.E.M., and the number of samples is shown in parentheses.

DURATION OF EXPERIMENT (min)	MASS OF ANIMALS (g)	RADIOACTIVITY REMAINING IN THE GUT	RADIOACTIVITY REMOVED FROM THE GUT		
			TCA precipitable	VASCULAR COMPARTMENT non-TCA-precipitable	VISCERA AND CARCASS
15 (10)	32.3 \pm 1.0	819.7 \pm 13.6	2.0 \pm 0.5	2.5 \pm 0.5	175.8 \pm 13.7
30 (10)	31.8 \pm 1.3	707.3 \pm 31.2	10.4 \pm 1.2	3.2 \pm 0.7	279.1 \pm 31.3
45 (10)	32.1 \pm 1.1	674.8 \pm 26.7	19.7 \pm 1.8	6.4 \pm 0.4	299.1 \pm 26.9
60 (13)	30.6 \pm 0.7	699.6 \pm 14.3	45.1 \pm 5.5	8.9 \pm 1.0	246.4 \pm 15.4
120 (16)	33.8 \pm 0.8	552.3 \pm 16.2	188.4 \pm 7.8	21.2 \pm 3.2	238.1 \pm 12.6
180 (11)	31.9 \pm 0.8	417.8 \pm 18.7	315.0 \pm 23.2	12.7 \pm 1.5	254.5 \pm 18.1
210 (6)	27.3 \pm 1.1	371.5 \pm 34.7	344.4 \pm 37.5	15.2 \pm 1.3	268.9 \pm 33.4
255 (6)	35.2 \pm 0.6	346.3 \pm 27.3	316.4 \pm 36.6	14.1 \pm 0.7	323.2 \pm 20.6
360 (6)	33.7 \pm 0.3	134.3 \pm 32.9	358.5 \pm 14.3	10.8 \pm 1.3	520.5 \pm 19.6

FIG. 6.3

The distribution of radioactive material with time in the vascular compartment and the viscera plus carcass, and its removal from the ligated proximal small intestine after the intraluminal injection of 1000 μg of labelled rat IgG (0.2 ml)

○ μg . removed from gut
□ μg . in viscera + carcass
▽ μg . in vascular compartment, [TCA ppt.]



constant for at least the next two-and-a-half hours, and represents 35% of the dose injected into the gut.

Figure 6.3 shows that immediately after intraluminal injection, there is a very rapid removal of radioactive material from the intestine. After only 30 minutes, 30% (300 μ g) of the injected dose has been removed, most of which is passed to the viscera and carcass. After this initial half hour period, the removal rate decreases, and over the next five-and-a-half hours IgG is removed from the intestine at a constant rate of 104 μ g per hour. In contrast to this, the passage of radioactive material to the viscera and carcass ceases after 30 minutes. There is no further input of radioactivity to these tissues until three-and-a-half hours after injection. This second period of accumulation of radioactivity in the viscera and carcass coincides with the level of TCA-precipitable IgG in the serum reaching its maximum value. Over the next two-and-a-half hours, radioactive material is passed to the viscera and carcass at a rate of 101 μ g per hour.

Table 6.4 and Fig. 6.4 show the uptake kinetics of a 250 μ g dose of IgG in 0.2 ml, injected into the proximal small intestine. This study, as can be seen from an examination of Fig. 6.1, examined the uptake of a sub-saturating dose of IgG, whereas in the previous study using a 1000 μ g dose, the intact transport mechanism was overloaded.

The results illustrated in Fig. 6.4 show that there is a slow removal of radioactivity from the intestine in the first few minutes after intraluminal injection of labelled IgG. Radioactive material is removed from the gut at a fairly constant rate of about 40 μ g per hour over the entire five hour duration of the experiment. During most of this period, TCA-precipitable radioactivity accumulated in the vascular compartment at a rate equivalent to the removal of radioactivity from the intestine.

FIG. 6.4

The distribution of radioactive material with time in the intestinal wash and wall, the vascular compartment and the viscera plus carcass, after the intraluminal injection of 250 μg of labelled rat IgG (0.2 ml) into the ligated proximal small intestine.

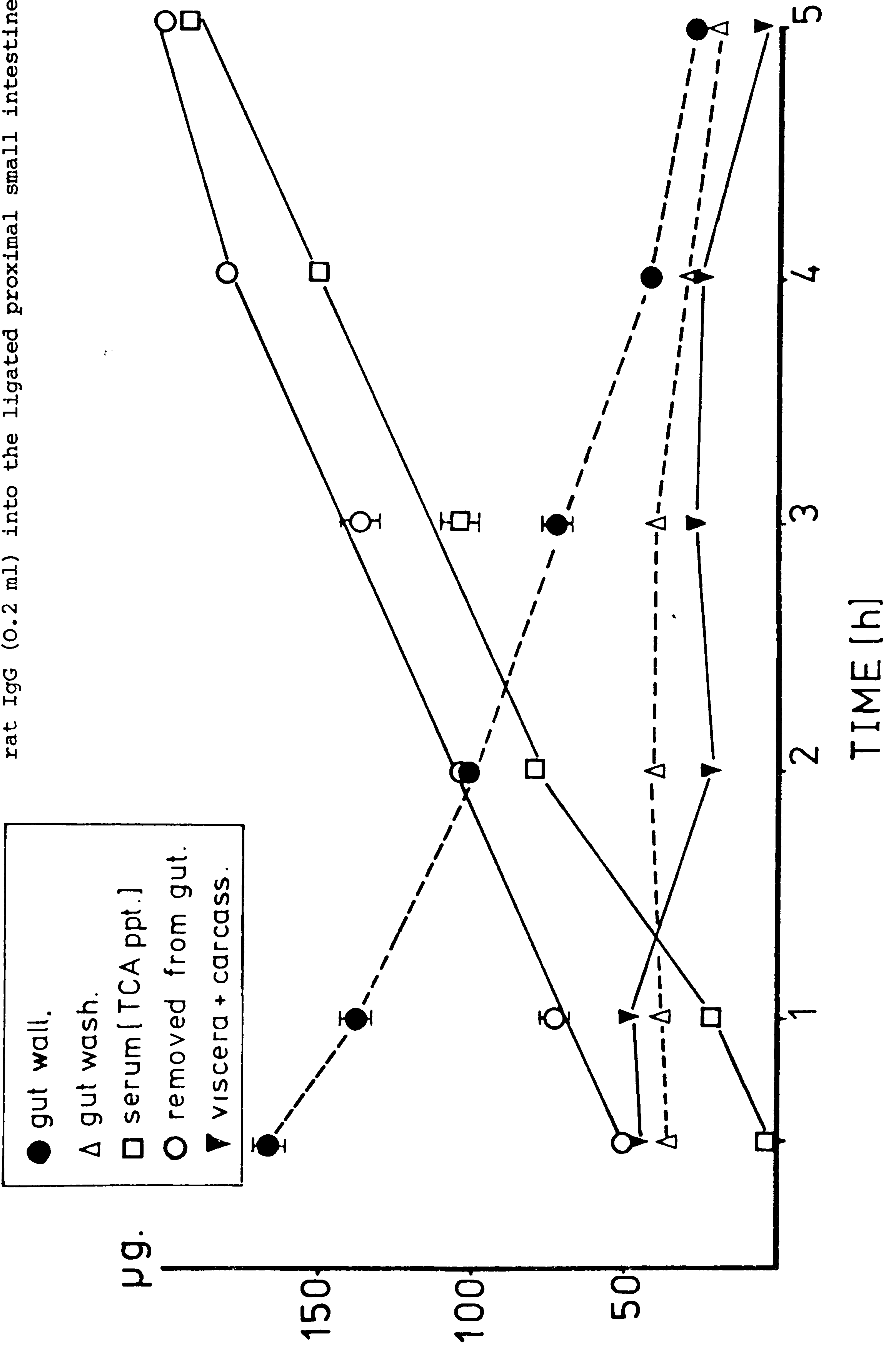


TABLE 6.4

The distribution of radioactive material with time in the intestine, vascular compartment and carcass plus viscera after the intraluminal injection of 250 µg of labelled rat IgG into the ligated proximal small intestine. The results, expressed as µg equivalents of protein, are given as mean values ± S.E.M. The number of samples is shown in parentheses.

DURATION OF EXPERIMENT (min)	MASS OF ANIMALS (g)	RADIOACTIVITY REMAINING IN GUT		RADIOACTIVITY REMOVED FROM GUT		
		IN GUT WALL	IN GUT WASH	TCA-precipitable	VASCULAR COMPARTMENT	VISCERA and CARCASS
30 (6)	34.7 ± 0.3	165.1 ± 5.7	36.2 ± 1.3	3.1 ± 0.6	1.4 ± 0.2	44.2 ± 5.5
60 (6)	39.1 ± 0.6	137.7 ± 4.6	39.4 ± 4.3	22.7 ± 2.7	0.9 ± 0.1	49.3 ± 3.7
120 (5)	34.9 ± 0.6	103.9 ± 4.9	41.7 ± 3.5	79.9 ± 3.2	2.1 ± 0.4	22.4 ± 2.9
180 (6)	37.3 ± 0.2	72.3 ± 4.8	40.7 ± 1.8	104.3 ± 5.8	5.2 ± 0.4	27.5 ± 3.4
240 (6)	33.2 ± 0.6	41.6 ± 2.9	27.4 ± 0.6	150.7 ± 3.0	3.7 ± 0.3	26.6 ± 2.3
300 (5)	33.6 ± 0.8	27.5 ± 1.9	21.3 ± 2.5	193.7 ± 7.5	3.2 ± 0.4	4.3 ± 6.8

Half an hour after injection, about 14.5% of the intraluminally administered dose remained in the gut wash, although only 19.5% of the dose had been removed from the gut. The bulk of the dose (66%) was present in the intestinal wall. Over the next four-and-a-half hours, the radioactivity which had accumulated in the enterocytes was transmitted to the vascular compartment, mainly as TCA-precipitable material. After this rapid initial uptake of IgG from the intestinal lumen into the enterocytes, the cells did not appear to remove any more radioactivity from the luminal contents until three hours after injection.

DISCUSSION

The saturability of the uptake mechanism for intact IgG in the suckling rodent intestine was first demonstrated by Halliday (1958) using anti-*Salmonella* agglutinins. The results presented in Fig. 6.1 and Table 6.1 are in full agreement with these findings, and should provide a more precise estimate of the saturability. A dose of 500-750 µg of labelled rat IgG in 0.2 ml was sufficient to saturate the capacity of the proximal small intestine to transport intact IgG to the vascular compartment.

The Brambell hypothesis (1970) stated that homologous and heterologous IgG present in the intestinal lumen was absorbed pinocytotically by the enterocytes. IgG molecules were assumed to bind to Fc-receptors located on the walls of the pinocytotic vesicles which subsequently fused with a lysosome. The receptor bound molecules were considered to be protected from degradation within the lysosome, whereas material in the lumen of the lysosome was digested. If the concentration of the IgG in the intestinal lumen was great enough, the numbers of IgG molecules would exceed the numbers of available receptors, and non-receptor bound IgG would be incorporated into the pinocytotic vesicles and consequently

degraded. Thus a major prediction of the hypothesis was that as the concentration of the intraluminally injected dose was increased, so the proportion of the dose removed from the gut as IgG breakdown products, would also increase. Conversely the proportion of the dose removed from the gut as intact IgG would decrease. The results presented in Fig. 6.2 and Table 6.2 clearly show these predictions are valid, since it has previously been demonstrated that the radioactive material present in the viscera and carcass represents IgG breakdown products (see Chapter 4).

These findings concerning the variation of IgG breakdown with administered dose concentration are in agreement with previous studies by Hemmings (1975a) and I. G. Morris (1976), although Hemmings (1976) subsequently claimed to be unable to demonstrate this phenomenon. I. G. Morris interpreted his results as being compatible with the Brambell hypothesis. The results of the present study are equally compatible with the predictions of that hypothesis.

Table 6.2 also demonstrated that the proportion of the dose removed from the gut was independent of the concentration in the 0.5 to 10.0 mg/ml range. The implication of this is that equal volumes of the IgG solution were endocytosed by the cells of the proximal small intestine, irrespective of the IgG concentration. This strongly suggests that the initial pinocytotic uptake of IgG from the gut lumen may be a non-selective process.

The pattern of IgG transmission from the proximal small intestine, as represented in Fig. 6.3 and Fig. 6.4, revealed several interesting features of the mechanisms involved.

From Fig. 6.4 it can be seen that immediately after IgG injection there was a rapid, though short lived, phase of IgG endocytosis by the enterocytes. Once the cells had loaded up with radioactive material, no further absorption of the luminal contents occurred for the next three

hours in the ligated region of intestine. The radioactive material endocytosed by the enterocytes immediately after injection was gradually released into the vascular compartment.

Figure 6.3 shows that half an hour after the injection of a 1000 μg dose of labelled IgG, 30% of the radioactivity was removed from the gut. Almost all of this material was associated with the viscera and carcass, which from previous studies is known to represent low molecular weight IgG breakdown products (see Table 4.3). After this initial half hour period, IgG digestion appeared to cease, and the rate of removal of radioactivity from the gut fell to a level of 104 μg per hour.

During the initial phase of rapid endocytosis by the enterocytes, a considerable amount of non-receptor bound IgG must have been internalized, since the 1000 μg dose was more than sufficient to saturate the receptor system (Table 6.1). This unbound IgG was then degraded by the digestive mechanisms within the enterocytes. The breakdown products of the digestion could readily pass out of the cells and into the viscera and carcass via the vascular compartment. This would account for the very rapid removal of 300 μg of radioactive material from the gut in the first 30 minutes, and the subsequent appearance of this material as IgG fragments in the viscera and carcass.

Once all of the non-receptor bound IgG in the enterocytes has been digested, and assuming that there is no further endocytosis of material from the intestinal lumen, one would expect the input of radioactivity to the viscera and carcass to terminate. Figure 6.3 clearly shows this to be the case.

From this point in time onwards, almost all of the radioactivity transported by the enterocytes accumulated in the vascular compartment as TCA precipitable material, which has been shown by ultracentrifugational analysis to represent 7S molecules (Fig. 4.2). From the foregoing

discussion and from Fig. 6.3, it is evident that the transcellular transport of intact IgG is a much slower process than its digestion and removal from the cell as breakdown products.

When the concentration of the IgG dose injected into the gut was insufficient to saturate the receptor system, the initial phase of rapid IgG digestion and removal of breakdown products to the viscera and carcass was absent (Fig. 6.4). This was presumably a consequence of most of the endocytosed material binding to receptors and thus being protected from digestion.

Intact IgG gradually accumulated in the vascular compartment, and as shown in Fig. 6.3 reached a maximum level of about 350 μ g, three and a half hours after injection. Concurrent with the achievement of this plateau, there was a renewed passage of IgG breakdown products to the viscera and carcass. A possible explanation for this observation is that the enterocytes switched over from transmitting intact IgG to digesting the protein and releasing breakdown products into the vascular compartment. This may be the result of an unavailability of Fc-receptors at the apical cell surface while they were in the process of being recycled. The rate of removal of radioactivity from the gut has been shown to be much greater via the digestive pathway, however as can be seen from Fig. 6.3, the rate remained constant over this period. Thus it seems unlikely that this proposed change in the pattern of IgG processing by the enterocytes can account for the renewed accumulation of breakdown products in the viscera and carcass.

However these observations do fit in with the concentration catabolism effect which is characteristic of gamma-globulins (Fahey and Robinson 1963; Waldmann and Strober 1969). This effect refers to the observation that as the concentration of IgG in the vascular compartment rises by endogenous production or by infusion, the fractional catabolic rate

increases until a limiting concentration is reached (Waldmann and Jones 1973). This phenomenon is unique to IgG among the immunoglobulins, and it can not be explained by shifts of protein between compartments nor is it the result of induction of catabolic enzymes (Waldmann and Strober 1969). Both the concentration-catabolism effect and the intestinal transmission of intact IgG in the neonatal rat gut show IgG specificity, involve all four subclasses of IgG (Morrell *et al* 1970), are mediated through the Fc terminal of the IgG molecule (Fahey and Robinson 1963) and have similar species specificity.

A saturable system of protective Fc-receptors, specific for IgG, has been proposed as a possible explanation for the concentration-catabolism effect (Brambell, Hemmings, Morris 1964; Waldmann and Strober 1969). This model involves the isolation of a fraction of the circulating IgG into a catabolic pool by means of pinocytosis. The IgG molecules become attached to a limited number of protective receptors, and this bound IgG is eventually returned to the circulation. All unbound IgG however, is digested. At low serum concentration most isolated IgG molecules would be protected and returned to the circulation, producing a long survival of the protein, whereas at high serum concentration the converse would be true. In support of this hypothesis, high molecular weight IgG complexes have been observed in homogenates of the eviscerated carcasses of germ-free mice four hours after the intravenous administration of labelled IgG (Waldmann and Jones 1973).

Thus the concentration-catabolism effect is similar to the intestinal transport of IgG in that it involves competition for a limited number of saturable membrane receptors that are specific to IgG and that in some way protect this molecule from catabolism.

CHAPTER 7

SUBCELLULAR FRACTIONATION STUDIES

INTRODUCTION

Ever since Brambell first proposed his hypothesis to account for the transcellular transport of intact immunoglobulins (Brambell 1958) there has been considerable controversy concerning the fate of these macromolecules within the cell. The modified hypothesis (Brambell 1970) predicted that endocytic vesicles formed at the plasma membrane contained not only receptor-bound protein, but also non-receptor-bound luminal protein which was fortuitously included during endocytosis. These vesicles subsequently fused with lysosomes, which resulted in the degradation of the non-receptor-bound material. The receptor-bound protein was eventually expelled at the basolateral membrane by exocytosis.

More recently, it has been suggested that coated vesicles are involved in the receptor mediated endocytosis of immunoglobulins (Rodewald 1973, 1980a; Wild 1975). The clathrin coat surrounding these vesicles has been alleged to prevent their fusion with lysosomes, and thus afford protection to the receptor bound protein.

The work of Hemmings (1975a, 1976) has challenged the Brambell hypothesis at an even more fundamental level. He has stated that receptors may not be involved at all in the selective transport of immunoglobulins, and that all proteins are adsorbed with equal facility onto the sticky glycocalyx and are then pinocytosed. Once inside the cell the vesicles rupture, so releasing the protein into the cytoplasm. Selection operates at the basolateral membrane by means of a diffusion carrier system.

In order to test the validity of these various models a series of cell fractionation experiments were performed in an attempt to identify the organelles actually involved in IgG transport across the cell.

METHODS

Isolation of IgG complexes

Differential and rate zonal centrifugation techniques were used in order to examine the formation of IgG complexes after injection of the immunoglobulin into the ligated proximal small intestine. One hour after injection of 0.2 ml of ^{125}I IgG into the duodenum, the animal was killed and the proximal intestine removed. The mucosa was squeezed out of the gut, using a metal spatula, and weighed. The mucosa was suspended in isotonic MES buffer (pH 6.0), 5 ml per gram of tissue. The buffer was composed of the following salts; and has an osmolality of 300 m Os/L.

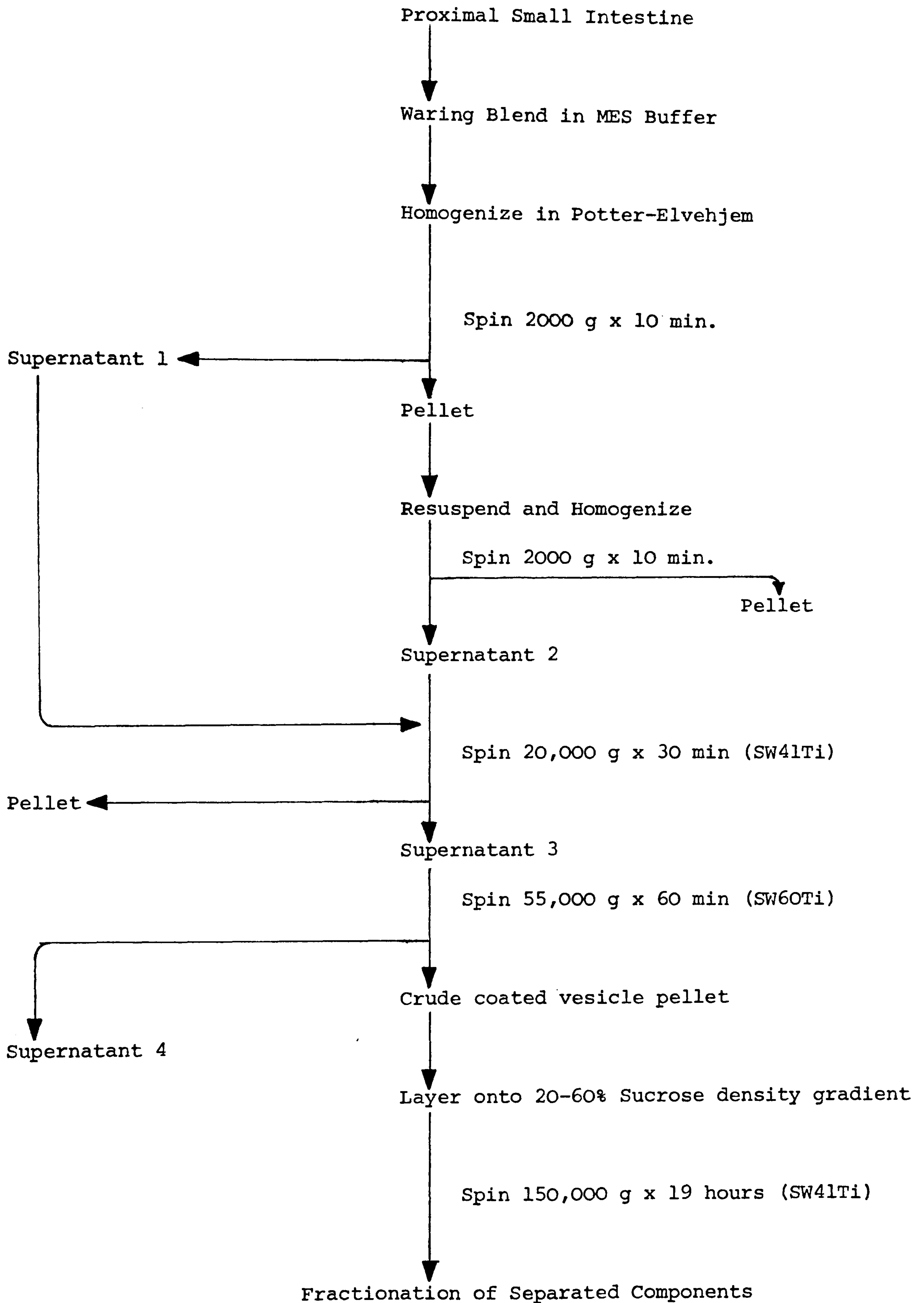
NaCl	6.89 g/l
KCl	0.35 g/l
CaCl ₂	0.37 g/l
MgSO ₄	0.30 g/l
Na ₂ HPO ₄	0.19 g/l
MES	5.33 g/l

This mucosal suspension was broken up using a top-drive Waring blender and then centrifuged at 300g for five minutes in order to pellet down the intestinal cells. The supernatant (S_1), which represented material not bound to the cells, was removed and the pellet was weighed and resuspended in twice its volume of the MES buffer. This was homogenized for five minutes in a motor driven Potter-Elvehjem homogenizer at full speed. Samples (300 μl) of the supernatant (S_1) and the mucosal cell homogenate were layered onto linear 10-60% (w/w) sucrose density gradients, and centrifuged for 24 hours at 205,600g at 4°C in a Beckman SW.41 Ti rotor. After centrifugation, the gradients were fractionated as described previously.

Isolation of coated vesicles

Using centrifugation procedures similar to those employed by Pearse (1975), an attempt was made to isolate coated vesicles from the rat proximal small intestine one hour after an intraluminal injection of ^{125}I labelled rat IgG. The purification scheme is shown in Fig. 7.1. Groups of 16 animals were operated upon, and at autopsy the regions of intestine which had received the radioactive protein were removed and weighed. The intestines were cut up into small pieces, placed in 5 ml of ice cold buffer (0.1 M MES pH 6.2, 1 mM EGTA, 0.5 mM Mg Cl₂, 0.02% sodium azide) per gram of tissue, and macerated in a Waring blender at full speed for 30 seconds. The macerate was homogenized in a Potter-Elvehjem homogenizer and the resulting suspension was centrifuged at 2000 g for ten minutes to remove large cellular debris. The supernatant (S₁) was kept on ice while the pellet was resuspended, homogenized and centrifuged again at 2000 g for ten minutes. The pellet was discarded, and the pooled supernatants (S1 + S2) were centrifuged at 20,000 g for thirty minutes (SW 41 Ti rotor). The supernatant (S3) was then further centrifuged at 55,000 g for an hour (SW 60 Ti rotor) to obtain a crude coated vesicle pellet. This was resuspended in a small volume of buffer, layered onto linear 20-60% (w/w) sucrose density gradients in 13.2 ml polyallomer tubes (Sw 41 Ti rotor), and centrifuged at 50,000 g for 19 hours. After centrifugation the gradients were fractionated and assayed for protein and radioactivity as described previously, and visualized by electron microscopy, using negative staining.

FIGURE 7.1 Purification Scheme for coated vesicles

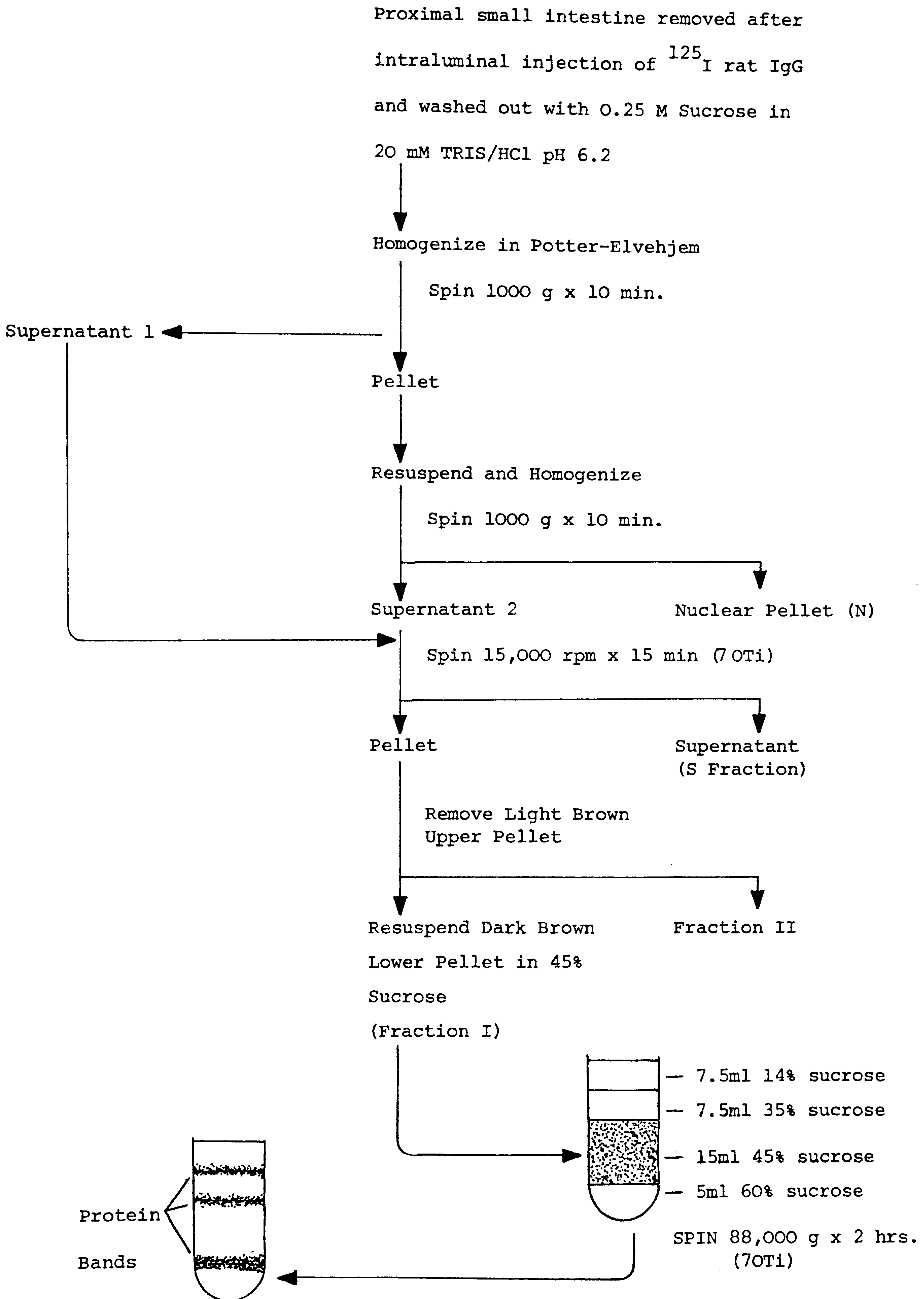


Lysosome purification

An enriched lysosome preparation was produced from the proximal small intestine by means of differential and rate zonal centrifugation procedure (Fig. 7.2). A 0.2 ml dose of ^{125}I labelled rat IgG was injected intraluminally, and after time intervals ranging from 2-30 minutes the animals were killed. At autopsy the proximal small intestine was removed and washed out with 5 ml of ice cold 0.25M sucrose in 20 mM TRIS/HCl buffer at pH 6.2. The gut tissue was homogenized in ten volumes of buffer by means of a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 1000 g for ten minutes, and the supernatant (S1) was removed and kept on ice. The pellet was resuspended, homogenized and centrifuged again at 1000 g for ten minutes. The supernatants were pooled and centrifuged for 15 minutes at 15,750 g (60Ti), producing a pellet with two well defined layers. The light brown upper layer was removed by washing carefully with the buffer, and the remaining dark brown pellet was resuspended in 15 ml of 45% sucrose in 20 mM TRIS/HCl pH 6.2. This suspension was placed in the 45% sucrose band of a discontinuous 14-60% sucrose density gradient (see Fig. 7.2) and centrifuged for two hours at 88,000 g (70Ti, rotor). The gradient was fractionated as described previously and the fractions were assayed for protein, radioactivity and enzyme activity.

The radioactivity present in the enriched lysosome preparation produced by this method, was subsequently examined by means of gel filtration, and the radioactivity in each of the fractions produced was measured.

FIGURE 7.2 Lysosomal purification scheme



Gel filtration

Gel filtration was performed on lysosomal material prepared as outlined previously (see Fig. 7.2). 0.5 ml samples of this material were sonicated to disrupt the organelles, and then applied to a 2.6 cm. x 40 cm column of Sephacryl G 200 at room temperature, and eluted at a rate of 12 ml per hour with 20 mM TRIS/HCl buffer at pH 6.2. Constant volume fractions were collected by means of an LKB Ultrorac fraction collection system incorporating a flow cell Uvicord attachment which monitored absorbance at 280 nm. The fractions were also assayed for radioactivity.

The column was calibrated by running 10 mg samples of the following molecular weight markers (Combithek Calibration proteins II, Boehringer) as shown in Fig. 7.7;

PROTEIN	MOLECULAR WEIGHT
Ferritin	450,000
Catalase	240,000
BSA trimer*	210,000
Aldolase	158,000
IgG	156,000
BSA dimer*	134,000
BSA	67,000
Chemotrypsinogen	25,000
Cytochrome C	12,500

* Bovine serum albumin was converted to an oligomeric series by the method of Griffith (1972).

Enzyme Assays

1. Succinate dehydrogenase

Principal: In the presence of succinate in a buffered (pH 7.4) medium, the tetrazolium salt is reduced by succinate dehydrogenase to a

water-insoluble red dye. The dye can be extracted with ethyl acetate, and the absorbance at 490 nm measured.

Reagents:

a) Substrate: 55 mM potassium phosphate 0.11% (w/v)

5-(-p-indophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium

55 mM Sodium succinate

25 mM Sucrose

b) Stopping reagent: 10% (w/v) TCA

Procedure: To 0.9 ml of the substrate add 0.1 ml of the sample containing 0.2-0.5 mg of protein. Leave for 15 minutes at 37°C, then add 0.1 ml of TCA. Extract the reduced dye with 4.0 ml of ethyl acetate in a stoppered glass tube. The absorbance of the dye is measured at 490 nm. The extinction coefficient is 20.1×10^3 l/mole cm.

2. Alkaline Phosphatase

Principal: Upon hydrolysis of p-nitrophenyl phosphate by phosphatase, p-nitrophenol and inorganic phosphate are formed. When made alkaline, p-nitrophenol is converted to a yellow complex readily measured at 400-420 nm.

Reagents: a) Substrate: Sigma stock substrate solution (p-nitrophenyl phosphate).

b) Substrate buffer: 0.1 mol/litre glycine in 0.001 mol/litre magnesium chloride at pH 10.5

Chloroform was added as a preservative.

c) Stopping reagent: 0.02 N NaOH.

Procedure: Pipette 0.5 ml of substrate and 0.5 ml of substrate buffer into each tube, and equilibrate at 37°C. Pipette 0.1 ml of water into the blank, and 0.1 ml of the test samples into the other tubes. After 30 minutes add 10 ml of 0.02 N NaOH, and read the absorbance at

400-420 nm using the blank as the reference. Alkaline phosphatase units corresponding to this reading are determined from the calibration curve prepared by diluting p-nitrophenol with 0.02 N NaOH.

3. Acid Phosphatase

Principal: Upon hydrolysis of p-nitrophenyl phosphate by phosphatase, p-nitrophenol and inorganic phosphate are formed. When made alkaline, p-nitrophenol is converted to a yellow complex readily measured at 400-420 nm.

Reagents:

a) Substrate: Sigma stock substrate solution (p-nitrophenyl phosphate).

b) Substrate buffer: 0.09 mol/litre citric acid in 0.01 M HCl at pH 4.8.

Chloroform added as a preservative.

c) Stopping reagent: 0.1 N NaOH.

Procedure: Pipet 0.5 ml of citrate buffer into each tube. Pipette 0.2 ml of water into the blank, and 0.2 ml of sample into the other tubes. Incubate at 37°C for 30 minutes, and terminate the reaction by adding 6.0 ml of 0.1N NaOH to each tube. Read the absorbance at 400-420 nm using the blank as the reference. Acid phosphatase units corresponding to this reading are determined from the calibration curve prepared by diluting p-nitrophenol with 0.02N NaOH.

4. Cathepsin D

Principal: Haemoglobin is degraded by the enzyme, with the liberation of peptides soluble in the presence of trichloroacetic acid. The concentration of liberated peptides is determined by their absorption at 280 nm.

Reagents:

- a) Incubation mixture: 0.25 ml of 1.0M Sodium Formate buffer
(pH 3.0)
0.5 ml of enzyme sample
0.25 ml of 8% (w/v) Haemoglobin solution
- b) Stopping reagent: 3% (w/v) TCA

Procedure: Leave the incubation mixture for one hour at 37°C.

Terminate the reaction with 5 ml of 3% TCA. Incubate this mixture for a further ten minutes, then filter the mixture through Whatman No. 1 paper. Read the absorbance of the filtrate at 280 nm. One unit of Cathepsin D will produce an increase in A_{280} of 1.0 per minute per ml.

5. β -Galactosidase

Principal: O-nitrophenyl- β -D-galactoside is hydrolysed by the enzyme to o-nitrophenol. When made alkaline o-nitrophenol is converted to a yellow complex readily measured at 400-420 nm.

Reagents:

- a) Substrate: 2.5 mM o-nitrophenol- β -D-galactoside in 0.2M Na_2HPO_4 /0.1M Citrate buffer at pH 3.6.
- b) Stopping reagent: 0.4M Glycine/1.0M Na_2CO_3 buffer at pH 10.3.

Procedure: To 3 ml of buffer add 0.5 ml of substrate and 0.5 ml of enzyme sample. Incubate the mixture at 37°C for 60 minutes, and stop the reaction with 4.0 ml of 0.4M Glycine/1.0M Na_2CO_3 buffer. Read the absorbance at 420nm.

One unit of enzyme will hydrolyse 1.0 μ mole of O-nitrophenyl- β -D-galactoside to o-nitrophenol per minute.

6. β -Glucuronidase

Principal: p-Nitrophenyl- β -D-glucuronide is hydrolysed by the

enzyme to p-nitrophenol. When made alkaline p-nitrophenol is converted to a yellow complex readily measured at 400-420 nm.

Reagents:

a) Substrate: 0.97 ml of acetate buffer (0.57 ml of glacial acetic acid/100 ml adjusted to pH 4.5 with 1 N NaOH).

0.03 ml of 0.1M p-nitrophenyl- β -D-glucuronide.

b) Stopping reagent: 2.0 ml of 0.5N NaOH.

Procedure: To 0.97 ml of buffer, add 0.03 ml of substrate and 0.03 ml of sample. Incubate the mixture for 15 minutes at 37°C, and terminate the reaction by adding 2.0 ml of 0.5N NaOH. Read the absorbance at 400-420 nm. One unit of enzyme will liberate 1.0 μ mole of p-nitrophenol from p-nitrophenyl- β -D-glucuronide per minute at 37°C and pH 4.5

Electron Microscopy

The coated vesicle preparation produced by the purification scheme outlined in Fig. 7.1 was examined by electron microscopy. A drop of sample, delivered from a Pasteur pipette was mixed with a drop of 1M hexanediol in 0.01 M sodium phosphate at pH 6.2. A drop of this mixture was placed on a Formvar coated grid, negatively stained with 1% uranyl acetate and air dried. Electron micrographs were obtained using a Corinth microscope.

Sodium dodecyl Sulphate polyacrylamide gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed on samples of coated vesicles, prepared from the proximal small intestine by the purification procedure described in Fig. 7.1. The samples were run on cylindrical 5% polyacrylamide gels with a 5%

stacking gel according to the method of Payne (1976). The sample had a final protein concentration of 1-2 mg/ml and contained 0.0625M TRIS/HCl pH 6.8, 2% S.D.S., 5% 2-mercaptoethanol and 10% sucrose. The sample was heated in a boiling water bath for two minutes, then allowed to cool to room temperature. Bromophenol blue was added at a final concentration of 0.001 % as a marker, and the sample was applied to the top of the stacking gel. Electrophoresis was carried out at 8 mA per gel until the marker approached the bottom of the tube. The separated proteins were stained for two hours by immersion in 0.2% Coomassie brilliant blue R250 in 50% methanol in water to which 7% by volume of glacial acetic acid is added. The gels were destained in 7% acetic acid. Bovine serum albumin which had been converted to an oligomeric series by the method of Griffith (1972) was used as a molecular weight marker.

RESULTS

Separation of IgG complexes

The formation of IgG complexes in the cells of the proximal small intestine, one hour after the intra-luminal injection of labelled IgG, was examined by means of rate zonal ultracentrifugation. The results obtained by the fractionation, on 10-60% sucrose density gradients, of the radioactive material present in homogenates prepared from proximal enterocytes, are illustrated in Fig. 7.3a. The bulk of the radioactivity was located at the 25-30S region of the gradient. Thus most of the radioactivity present within the cells of the proximal small intestine after one hour has a molecular weight greater than the labelled IgG originally injected into the gut lumen.

In contrast, Fig. 7.3b shows the fractionation of the S1 supernatant, which represented the radioactive material which was not bound to the proximal enterocytes. This material did not appear to form a high

molecular weight complex and it migrated on the sucrose density gradient with a sedimentation coefficient of 7S.

Isolation of coated vesicles

Using a centrifugation procedure similar to that employed by Pearse (1975), an attempt was made to isolate coated vesicles from the proximal small intestine, one hour after the intraluminal injection of labelled rat IgG. Analysis of the crude coated vesicle pellet by rate zonal ultracentrifugation demonstrated the presence of a radioactive peak associated with material having a sedimentation coefficient of about 250 S (see Fig. 7.4), which is similar to the value reported for coated vesicles (Crowther *et al* 1976). This fraction was removed and examined by electron microscopy using negative staining techniques. Plate 7.1 shows particles with a polyhedral appearance and a diameter of approximately 70 nm. In both respects these particles resemble the material isolated by Pearse.

Further examination of the crude coated vesicle pellet by means of S.D.S. polyacrylamide gel electrophoresis revealed small quantities of a protein with an apparent molecular weight of 180,000 (see Plate 7.2).

Lysosome purification

A lysosome enriched fraction was prepared by the method outlined in Fig. 7.2. The protein content of the separated fractions was determined using the method of Lowry (1951) and the activities of the following enzymes were determined as markers for respective subcellular organelles; succinate dehydrogenase for mitochondria (Morre' 1971); alkaline phosphatase for plasma membranes (Sigma technical bulletin No. 104); β -galactosidase (Conchie 1959); acid phosphatase (Sigma technical bulletin No. 104); Cathepsin D (Barrett 1967) and β -glucuronidase (Robins *et al* 1968) for lysosomes.

The relative specific activity of these enzymes (defined as the ratio of the enzyme activity per mg of protein in a fraction to that in the homogenate), in each of the various fractions produced are shown in Table 7.1. It can be seen from this data that Fraction I had the highest relative specific activity of lysosomal, mitochondrial and plasma membrane enzyme markers. This material was taken and further fractionated by means of ultracentrifugation in a discontinuous sucrose density gradient, and the relative specific activity of the various enzyme markers was determined for each fraction. As shown in Fig. 7.5, fractions 3-5 displayed the highest relative specific activity for the lysosomal markers, whereas the mitochondrial and plasma membrane markers had been concentrated in fractions 1 and 8-11 respectively.

Using this cell fractionation technique, the distribution of radioactivity within the various fractions, was examined two to thirty minutes after the intraluminal injection of labelled rat IgG. As shown in Table 7.2, the relative specific radioactivity (defined as the ratio of the radioactivity per mg of protein in a fraction to that in the homogenate) was highest in fraction I.

Fraction I material obtained two minutes and 30 minutes after intraluminal IgG injection, was further purified by ultracentrifugation. Fractions 3-5, which had previously been shown to be enriched in lysosomes, were pooled and the organelles disrupted by sonication. Figure 7.6 shows the gel filtration analysis of the radioactive material associated with fractions 3-5, both two and 30 minutes after the intraluminal injection of labelled rat IgG. After two minutes the lysosomal fraction appeared to contain both intact IgG and IgG breakdown products with a molecular weight of approximately 32,000. However, after thirty minutes the lysosomal fraction appeared to contain only intact IgG.

Figure 7.7 shows the molecular weight calibration curve for the column.

TABLE 7.1 The distribution of enzymes in the lysosome purification technique. Absolute values are in mg for protein, and in units or moles for enzymes. E = the cytoplasmic extract; N = Nuclear fraction; I = Fraction I; II = Fraction II; S = Final supernatant.

	ABSOLUTE VALUE	PERCENTAGE VALUES					
		E+N	N	I	II	S	RECOVERY
Protein	175.6 mg	100	32.4	5.1	12.3	45.5	95.2
β Galactosidase	2.7×10^{-5} U	100	27.7	13.4	18.1	41.4	100.6
β Glucuronidase	9.0×10^{-5} U	100	40.9	11.4	10.3	56.8	119.4
Cathepsin D	0.13 U	100	22.4	12.4	17.0	38.1	89.9
Acid Phosphatase	720.3 U	100	26.7	6.3	14.7	39.1	86.8
Alkaline Phosphatase	1.97×10^3 U	100	18.7	33.4	25.9	31.3	109.3
Succinate Dehydrogenase	157.9×10^{-9} moles	100	35.7	16.0	14.1	39.7	105.5

	RELATIVE SPECIFIC ACTIVITY			
	N	I	II	S
β Galactosidase	0.86	2.63	1.47	0.91
β Glucuronidase	1.26	2.24	0.84	1.25
Cathepsin D	0.69	2.43	1.38	0.84
Acid Phosphatase	0.82	1.24	1.20	0.86
Alkaline Phosphatase	0.58	6.54	2.11	0.69
Succinate Dehydrogenase	1.12	3.14	1.15	0.87

TABLE 7.2 The relative specific radioactivity in the various subcellular fractions, 2-30 minutes after the intra-intestinal injection of labelled rat IgG.

TIME (mins)	RELATIVE SPECIFIC RADIOACTIVITY			
	N	I	II	S
2	0.52	1.81	0.62	1.46
5	0.59	1.71	0.56	1.43
15	0.73	1.47	0.70	1.33
30	0.52	1.89	0.83	1.26

FIGURE 7.3a

The ultracentrifugation on a linear 10-60% sucrose density gradient, of a homogenate prepared from the cells of the proximal small intestine, one hour after the intraluminal injection of ^{125}I labelled rat IgG. The radioactivity (r) and the % absorption at 280nm (uv) of each fraction represent the mean values from four experiments.

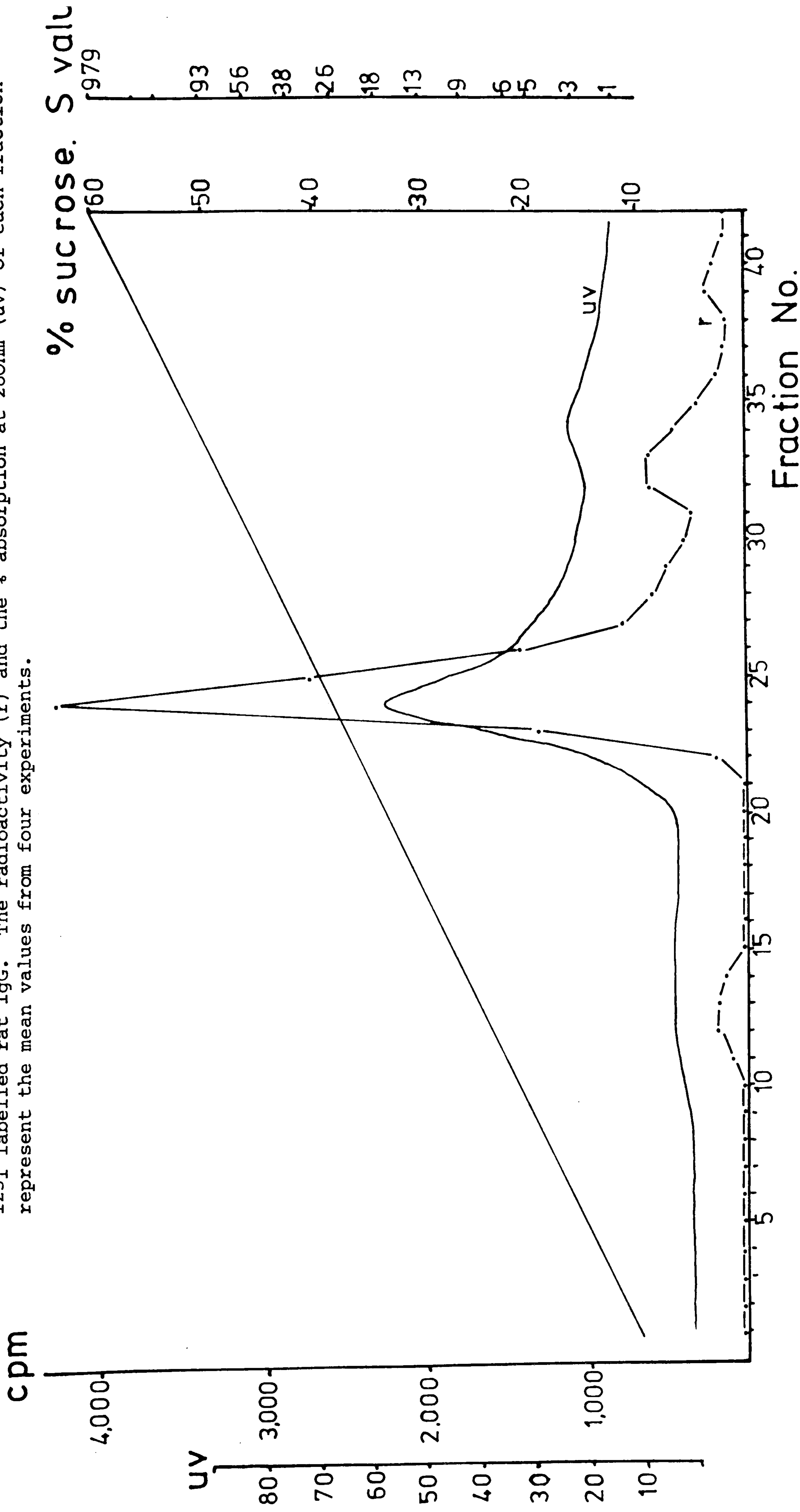


FIGURE 7.3b

The ultracentrifugation on a linear 10-60% sucrose density gradient, of the S_1 supernatant prepared from the proximal small intestine one hour after the intraluminal injection of ^{125}I labelled rat IgG. The radioactivity (r) and the % absorption at 280 nm (uv) of each fraction represent the mean values from four experiments.

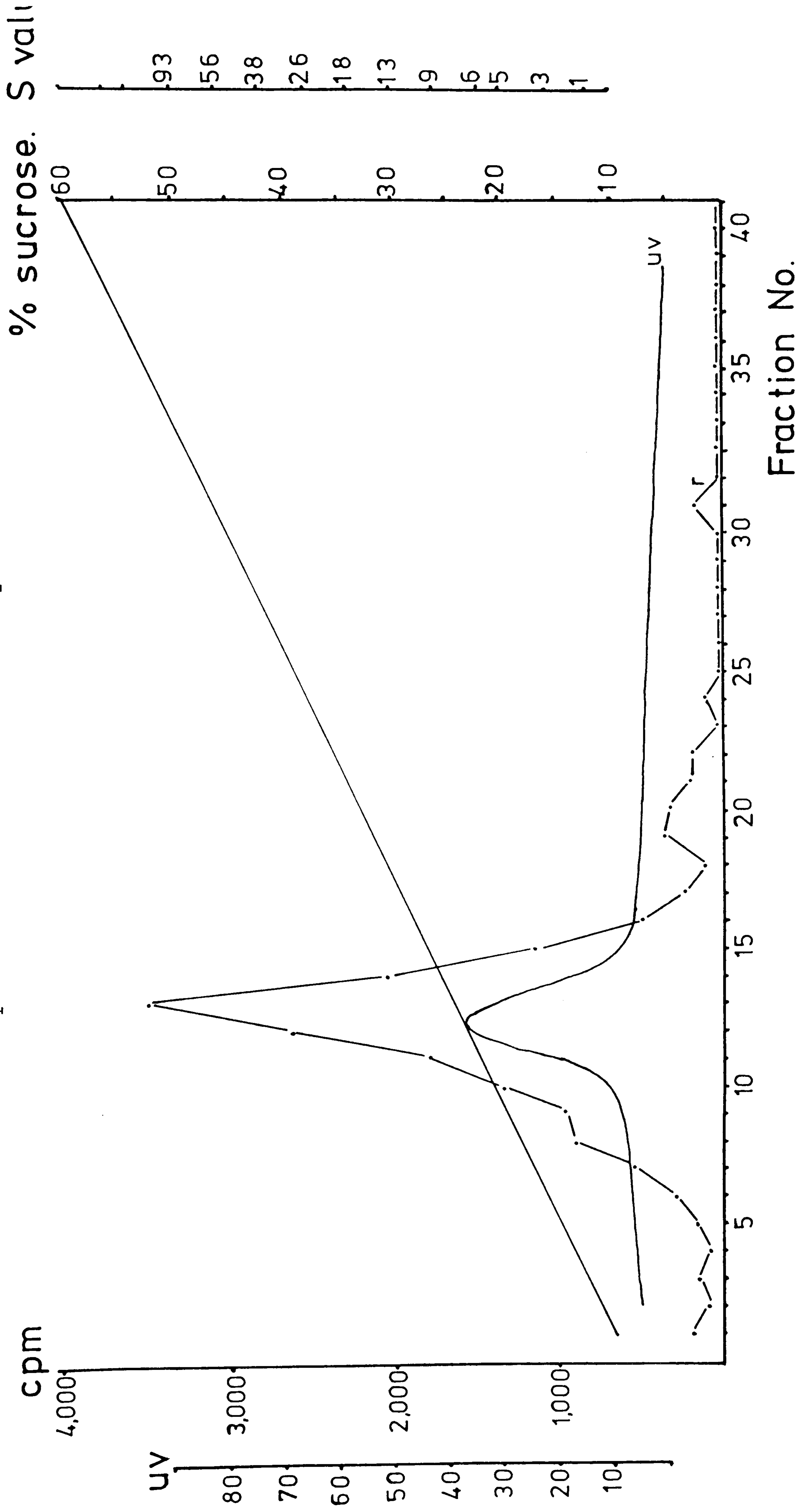


FIGURE 7.4

The ultracentrifugation on a linear 20-60% sucrose density gradient, of a crude coated vesicle pellet prepared from the proximal small intestine one hour after the intraluminal injection of ¹²⁵I labelled rat IgG. The radioactivity (r) and the % absorption at 280 nm (uv) of each fraction are shown.

CPM UV % Sucrose S value

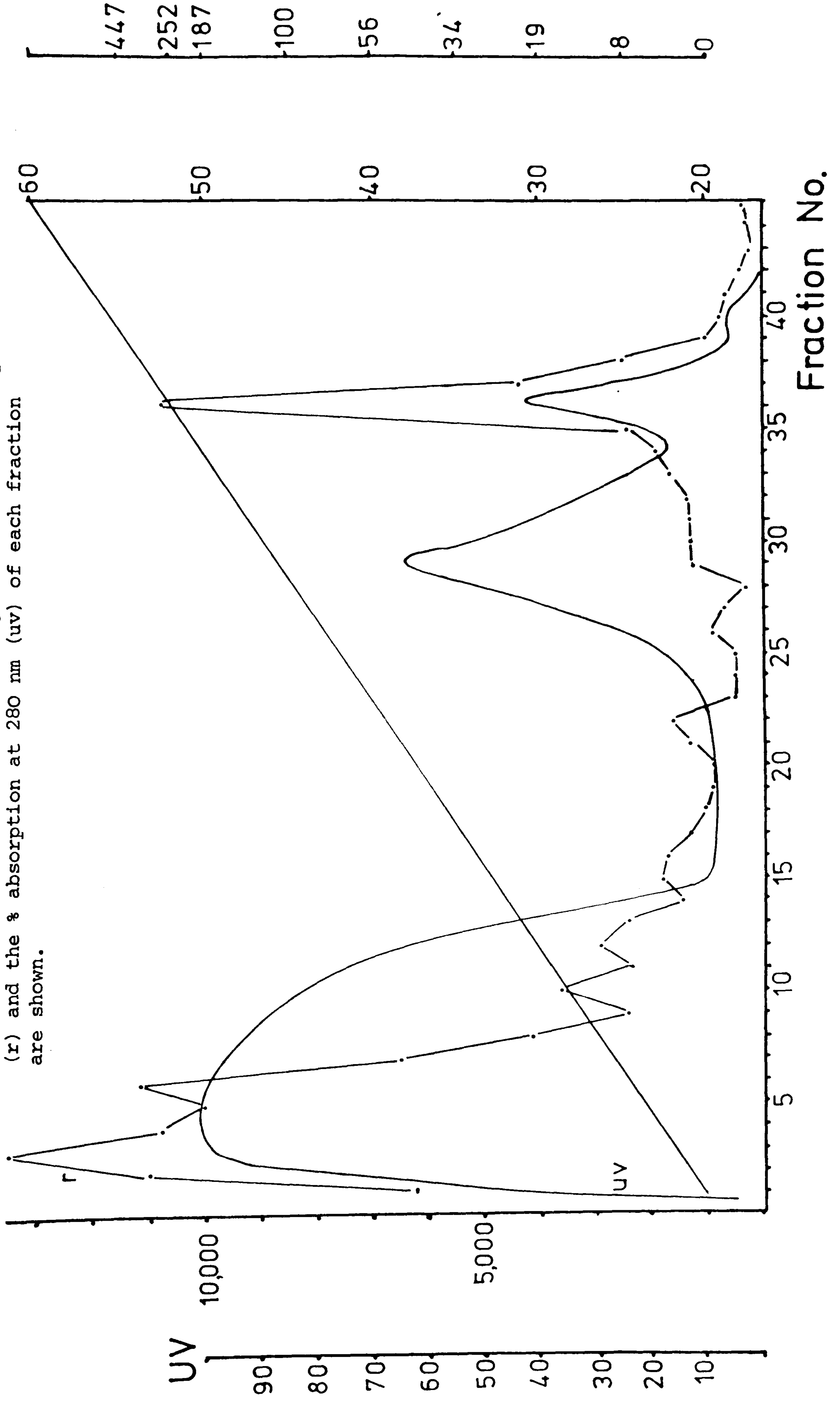
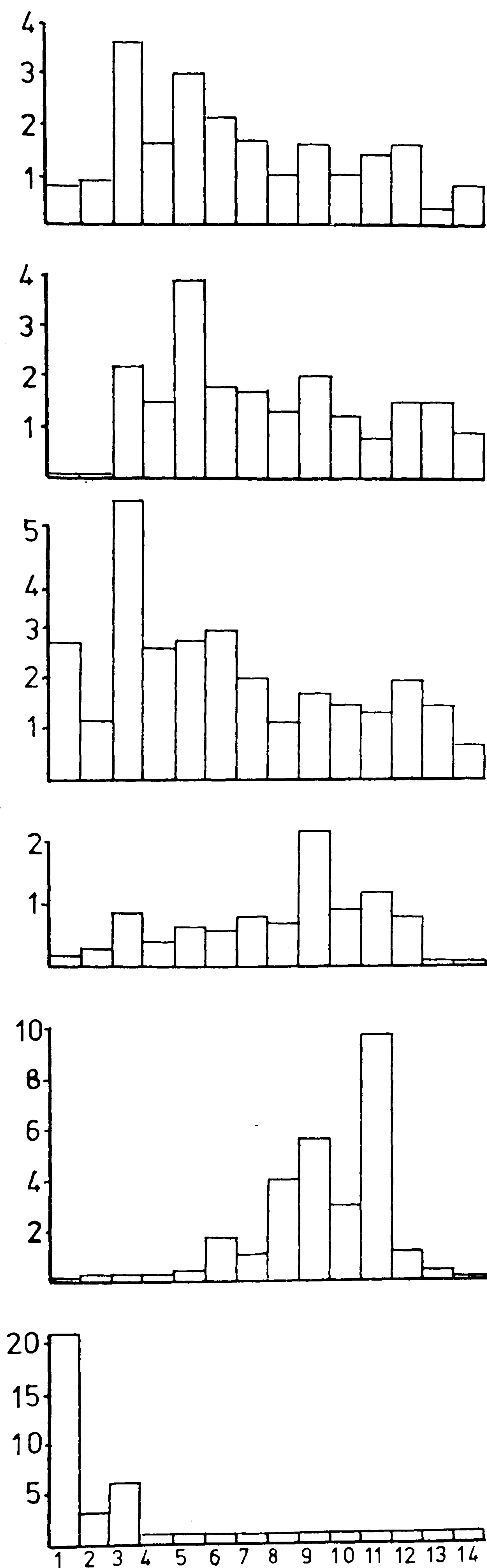


FIGURE 7.5
 The distribution of enzyme activity after the ultracentrifugation of the Fraction I pellet in a discontinuous 14-60% sucrose density gradient. Fraction 1 represents the bottom of the gradient, and Fraction 14 represents the meniscus.

RELATIVE SPECIFIC ACTIVITY



β galactosidase

β glucuronidase

Cathepsin D

Acid Phosphatase

Alkaline Phosphatase

Succinate Dehydrogenase

Fraction Number

FIGURE 7.6

The radioactivities of Sephacryl G200 eluate fractions of lysosomal contents, two and thirty minutes after the injection of 1 mg of ^{125}I labelled rat IgG into the proximal small intestine.

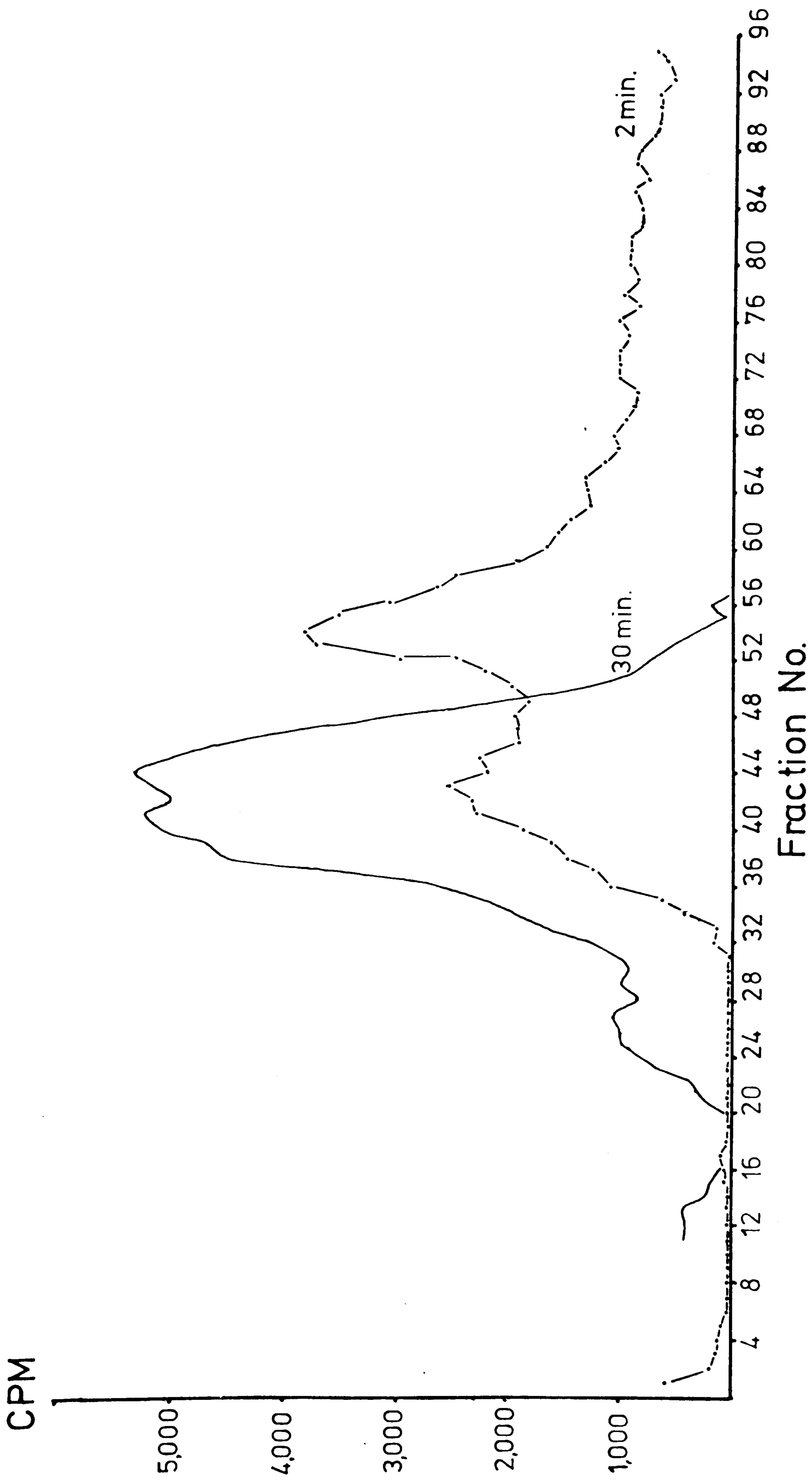


FIGURE 7.7 Molecular weight calibration curve for Sephacryl G200 column.

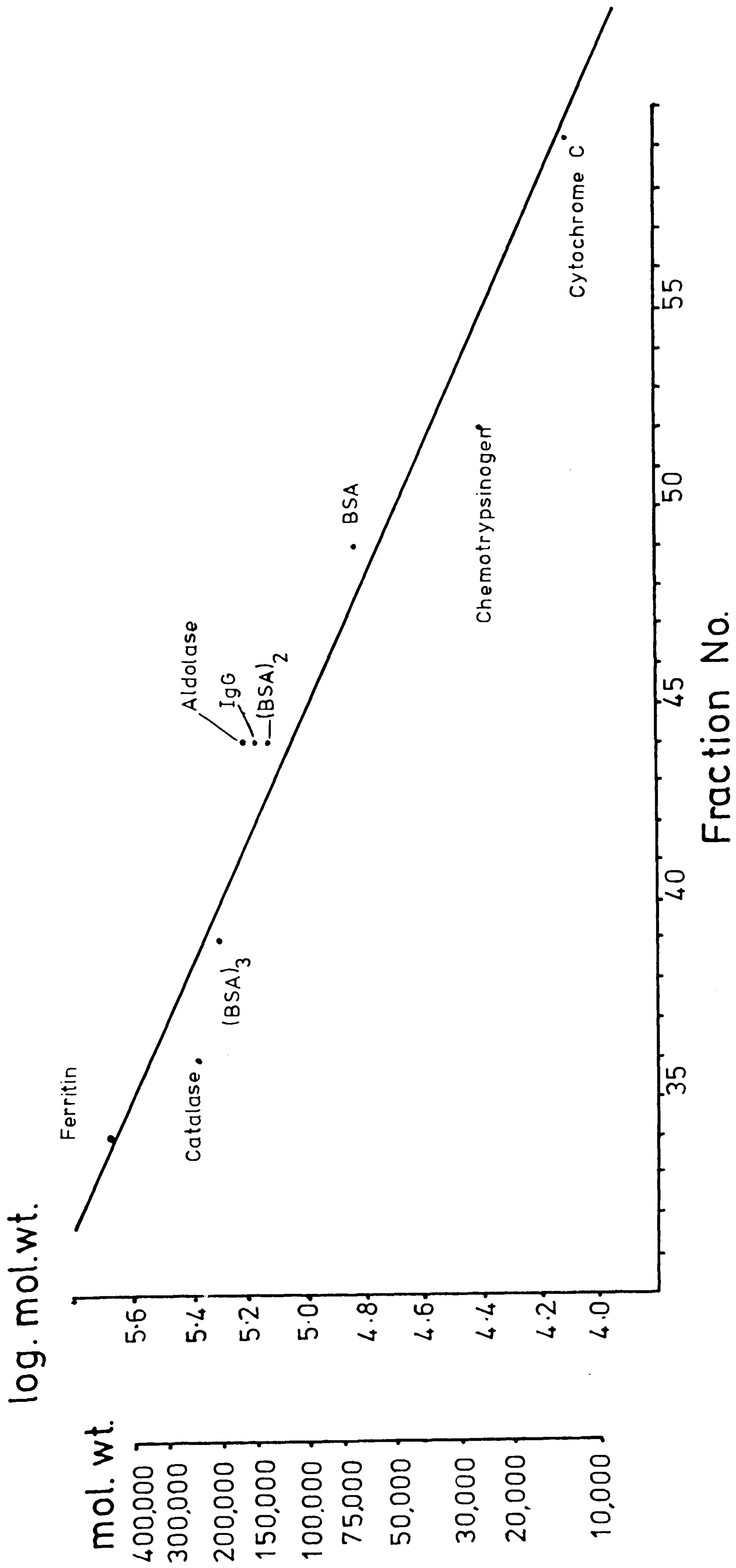


PLATE 7.1

Electron micrograph of the 250 S radioactive material recovered from the ultracentrifugation of the crude coated vesicle pellet on a 20-60% sucrose density gradient. The "coated vesicles" (arrowed) were negatively stained with 1% uranyl acetate. Magnification x 25,000.

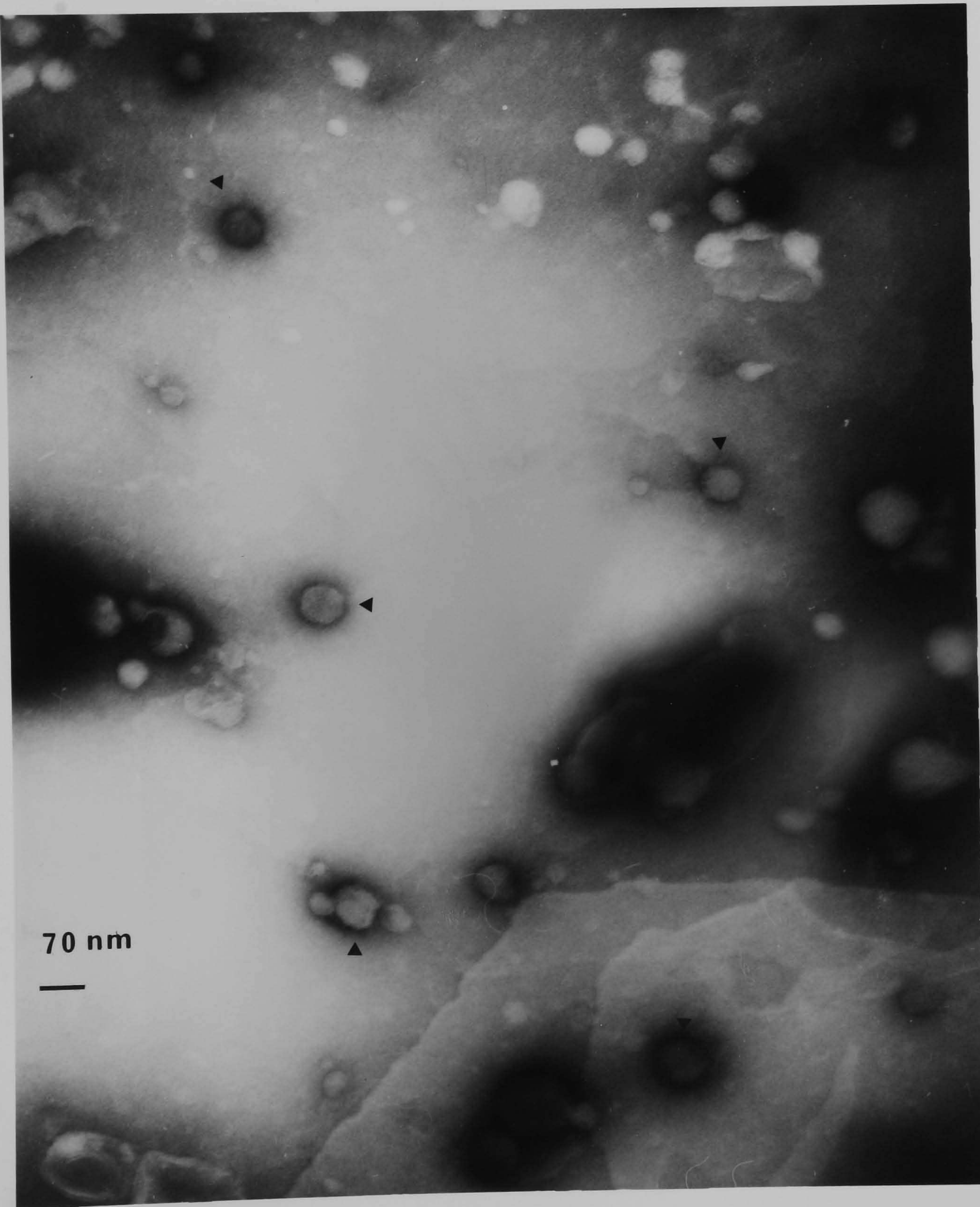
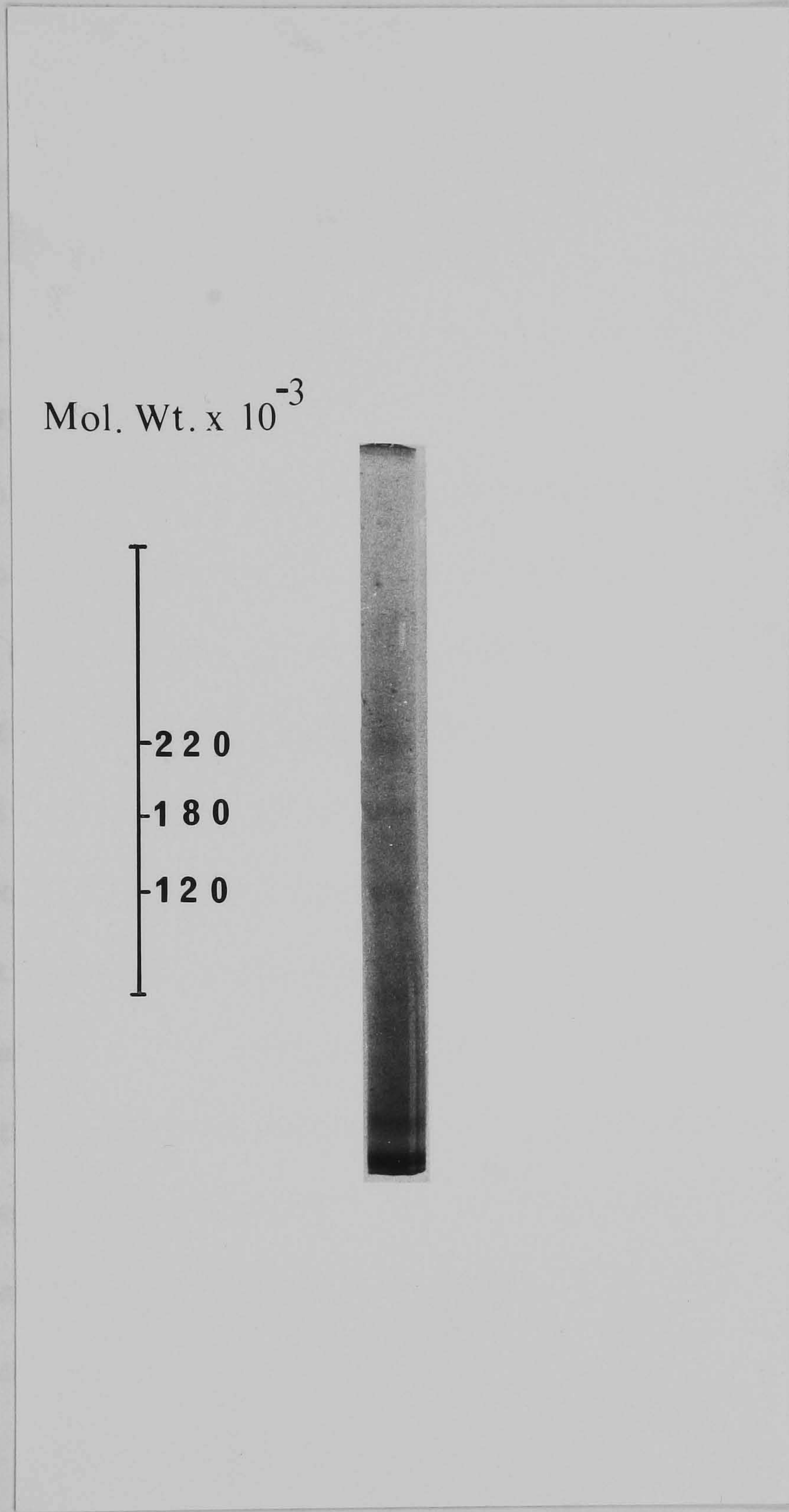


PLATE 7.2

S.D.S. polyacrylamide gel electrophoresis of the crude coated vesicle pellet.



DISCUSSION

The studies concerning the formation of IgG complexes in the wall of the proximal small intestine appeared to confirm the findings of Jones and Waldmann (1972), that these complexes migrated on sucrose density gradients with a sedimentation coefficient of 25-30S. This complexing of IgG molecules was only observed after the attachment of the IgG to the intestinal cells as shown in Fig. 7.3 a and b. After transport to the blood as shown previously (see Fig. 4.2) the IgG molecules are no longer associated with these high molecular weight complexes but are found to migrate as 7S molecules. These observations are consistent with the concept of an IgG receptor mechanism.

It has been claimed on the basis of morphological evidence, that antibody transport across the proximal cells of the small intestine occurs by means of a system of coated vesicles (Rodewald 1973). It was desirable therefore to attempt to isolate these organelles using the method previously developed by Pearse (1975). After numerous attempts to repeat the experiments performed originally by Pearse on porcine brain, it appeared that the young rat small intestine did not provide sufficient starting material for this method to be practically possible. Repeated examination of the material prepared by this method using S.D.S. polyacrylamide gel electrophoresis failed to demonstrate the presence of the 180,000 molecular weight protein called Clathrin which is claimed to be characteristic of these vesicles.

An attempt was made to increase the yield of coated vesicles at the expense of the purity of the preparation. A crude microsomal pellet was prepared from the intestinal mucosa one hour after the intra-luminal injection of labelled rat IgG. The pellet was resuspended and examined by means of sucrose density gradient ultracentrifugation. Figure 7.4 shows that a considerable amount of the radioactivity in the gradient was associated

with particles migrating with a sedimentation coefficient of about 250 S. This corresponds closely with the value for the sedimentation coefficient of coated vesicles which Crowther (1976) claims to be 220 S.

After fractionation of the gradient, the 250 S peak was examined by electron microscopy using negative staining techniques. This revealed polyhedral particles having a diameter of approximately 70 nm, which is within the expected range for coated vesicles. However the basket-like framework usually associated with these organelles was not clearly visible, and consequently some doubt may remain as to the precise nature of this material.

S.D.S. polyacrylamide gel electrophoresis of the crude coated vesicle pellet (Plate 7.2) revealed a faint band of protein with a molecular weight of 180,000. This protein, which has been named Clathrin, is considered to be the major constituent of the basket-like coat which is characteristic of coated vesicles. The fact that Clathrin was detected in the so called "crude coated vesicle pellet" (See Fig. 7.1) supports the findings of the electron microscope and ultracentrifugation studies. Thus on balance, the evidence presented appears to confirm the involvement of coated vesicles in the transcellular transport of IgG across the neonatal rat gut as proposed by Rodewald (1973).

A prediction inherent in the Brambell hypothesis is that intact IgG will be present in those lysosomes to which pinocytotic vesicles containing IgG fuse. In order to examine this prediction, a technique was developed for the preparation of a lysosome enriched fraction from the proximal small intestine, two minutes, and thirty minutes after the intraluminal injection of labelled rat IgG. The major technical difficulty encountered in this process was the tendency of the Fraction I pellet to bind together into a sticky mass which floated on top of preformed sucrose density gradients and self-forming Percoll gradients. Separation of the

Fraction I pellet was achieved by placing the resuspended pellet in the 45% sucrose band of a discontinuous 14-60% (w/w) sucrose density gradient, and centrifuging at 88,000 g for two hours. This procedure led to the formation of three distinct bands of protein in the gradient, two of which had floated upwards. As shown in Fig. 7.5 there was a concentration of lysosomal marker enzymes in fractions 3-5 of up to 5.5 times greater than in the original homogenate.

This enriched lysosomal fraction was prepared from the proximal small intestine after introducing labelled IgG into that region of the gut for periods of two and thirty minutes. The results summarized in Fig. 7.6 show that after a two minute incubation, the lysosomal fraction contained both intact IgG and IgG breakdown products. However, after a thirty minute incubation, no breakdown products could be detected.

From the kinetic studies presented in Chapter 6, it can be seen that the passage of breakdown products from the gut to the vascular system terminates approximately thirty minutes after the intraluminal injection of 1 mg of labelled rat IgG. The data presented in this study indicates that by this time, the products of IgG digestion have diffused out of the lysosomal system, leaving only intact IgG within the enterocytes which appears to be protected from the digestive mechanisms of the cell.

The relative specific activity of various enzyme markers was used as the criterion for the purity of the lysosomal preparation. However, there may have been contamination of the preparation from other cellular materials for which enzyme markers are not known, or were not used. So although the results seem to indicate the presence of intact IgG in lysosomes, which would confirm the Brambell hypothesis, it is possible that this IgG was contained in organelles which were co-purified with the lysosomes. Hence the interpretation of these results is unfortunately speculative.

CHAPTER 8

DISCUSSION

In this concluding chapter, the evidence which has been presented in this thesis will be examined to see if the data can provide fresh insights into the mechanisms of transcellular IgG transport in the suckling rat gut.

The validity of the quantitative results depends upon a number of factors:

- a) to what extent does ligation of the proximal small intestine alter the normal digestive physiology of this region of the gut?
- b) How accurate is the estimation of plasma volume?
- c) How sure can we be that TCA-precipitable radioactive material in the vascular compartment does in fact represent intact IgG, and not large IgG breakdown products?
- d) How justified is the statement that the bulk of the radioactivity present in the viscera and carcass represents IgG breakdown products?

Since all of the studies involving the transmission of IgG from the gut were performed on the ligated proximal small intestine, it was of interest to examine to what extent the operational procedure affected the proteolytic activity of the luminal contents. Table 5.1 shows that the protease level in the ligated gut was not significantly lower than in the unligated gut. In addition to this, the morphological studies of Rodewald (1973) have revealed that the integrity of the epithelial cells of the proximal small intestine was not altered by ligation. Thus the techniques employed were not excessively traumatic to the intestine.

In order to estimate the amount of radioactivity present in the whole vascular compartment, it was necessary to assay the activity of a 0.1 ml serum sample and to multiply this by the plasma volume. The

plasma volume per unit mass was determined by a radio-tracer dilution technique, and a value of 6.57 ml/100g body weight was obtained. This value was significantly higher than values previously reported for young rats (Belcher and Harriss 1957; Travnickova and Heller 1963; Morris and Morris 1976). This suggested that the radio-tracer used in this study was being cleared from the plasma very rapidly. This possibility was examined by extending the tracer mixing time, and by the use of ^{125}I PVP which is not catabolised. These methods confirmed the validity of the plasma volume value obtained (6.57 ml/100g). The discrepancies between this and previous estimates must therefore be due to differences in strain, age, weight or diet of the animals examined.

It has been demonstrated that substantial amounts of protein breakdown products with a molecular weight as low as 13,000, or a sedimentation coefficient of only 1S, could be precipitated with TCA (Jones 1979; Morris and Morris 1979). Is it justifiable therefore, to use TCA precipitation as a method of detecting intact IgG in the vascular compartment? It is clear from ultrafiltration studies (Table 4.3) that after the intraintestinal injection of labelled IgG, whether homologous or heterologous, almost all of the radioactivity present in the vascular compartment was associated with material exceeding 100,000 mol. wt. or less than 1000 mol. wt. Ultracentrifugation of serum samples in 5-25% (w/w) linear sucrose density gradients (Fig. 4.1 - 4.5) revealed that the radioactive material had a sedimentation coefficient of 7S, which was equivalent to that of an IgG standard. In both analytical procedures, there appeared to be no breakdown products of intermediate size which might have been precipitated by TCA. Consequently it is safe to conclude that almost all of the TCA precipitable radioactivity in the vascular compartment represents intact IgG molecules.

In the IgG transmission studies, the radioactivity which had been

removed from the gut and which could not be accounted for in the vascular compartment, was considered to be associated with the tissues of the viscera and carcass. Ultrafiltration analysis of the radioactivity present in the viscera and carcass homogenates was performed two hours after the intra-duodenal injection of both homologous and heterologous IgGs. These studies revealed that on average 64.5% of the radioactivity present in the viscera and 81.4% of the radioactivity present in the carcass had a molecular weight of less than 1000. These values are probably underestimates since it was not possible to remove by perfusion all traces of blood from the tissues prior to homogenization. Thus most, if not all of the radioactivity associated with these tissues represents the products of IgG digestion.

Digestion studies

In early studies it was considered that the immaturity of the digestive mechanisms in the neonatal gut, as demonstrated by Hill (1956), prevented the proteolysis of IgG destined for transport to the circulation. However the observation that intact transmission was accompanied by substantial degradation led workers to investigate the significance of the luminal and intracellular digestive properties of the gut.

Jones (1972), on examination of the fate of labelled IgG injected into the intestinal lumen, concluded that effective luminal proteolysis had occurred. In subsequent studies (1978) he revealed marked regional differences in proteolytic activity along the small intestine. These findings demonstrated that the lumen of the proximal small intestine had a much lower proteolytic capacity than the distal lumen. The data presented in this thesis (Fig. 5.1 and Fig. 5.2) confirmed this distribution of proteolytic activity.

Measurement of the levels of proteases present in luminal washings

indicated that in 14 day-old animals, the proteolytic activity, under assay conditions, remained constant from the pyloric end to the caecal end of the small intestine (Fig. 5.4). The differences between the proximal and distal regions under physiological conditions, as shown by Fig. 5.1 and Fig. 5.2 must therefore be due to the difference in pH between these two regions. The optimum pH for luminal proteolytic activity is eight (Jones 1972), and it has been shown that the pH of the proximal small intestine is 6.2 and the distal small intestine is 7.4 (Rodewald 1976c).

The large increase in luminal protease levels at 19 days of age (Fig. 5.4) may be a contributory factor in the termination of the transport of intact IgG.

Having demonstrated that a measurable degree of proteolysis had occurred in the lumen of the proximal small intestine, it was of interest to examine whether transmission could be enhanced by blocking luminal proteolytic activity. This was achieved by the addition of trypsin inhibitor to the injected dose of labelled IgG. Table 5.3 shows that inhibition of luminal proteolysis did not increase the amount of TCA-precipitable material transmitted to the vascular compartment. At the high IgG concentration the uptake mechanism was saturated (See Fig. 6.1) and consequently receptor availability was the limiting factor. However, at the low IgG concentration the uptake mechanism was not saturated and any luminal digestion of IgG should have resulted in a decrease in the transmission of intact IgG to the circulation. Such a decrease was not observed in the absence of trypsin inhibitor, indicating that luminal proteolysis did not affect IgG transmission significantly.

IgG transmission studies

The phenomenon of selection, which was first demonstrated by Bessis

and Freixa (1947a, b), has subsequently been reported by many workers. The evidence presented in the literature concerning the level at which selection operates is conflicting. There is strong morphological evidence in support of selection occurring at the cell surface, prior to internalisation. Rodewald (1973) demonstrated that ferritin conjugates of rat and bovine IgG were endocytosed by means of tubular and coated vesicles, whereas ferritin conjugated BSA or free ferritin did not enter the cell, nor appear in the intercellular spaces. He suggested that IgG was selectively absorbed at the apical cell surface by pinocytosis within tubular vesicles. The IgG is then transferred to coated vesicles, which carry the IgG across the cell and deposit it in the intercellular space with the aid of a pH shift (Rodewald 1976a, 1976b, 1980a).

Wild (1976) proposed a modification of the Rodewald model, and stated that both non-selective and selective uptake occurred in yolk-sac endodermal cells. Using fluorescent antibody techniques he demonstrated the non-selective uptake of human and bovine IgG by the apical tubular vesicles. These vesicles subsequently fused with lysosomes, and the internalized material was digested. Using fluorescently labelled anti-Cathepsin D antibodies, Wild demonstrated that Cathepsin D was present in macropinocytotic vesicles in association with human and bovine IgG. However, Cathepsin D could not be detected in and below the basement membrane. Wild concluded from these findings that phagolysosomes never discharged their contents into the intercellular space.

The transcellular transmission of intact IgG was accounted for by the selective uptake of IgG molecules by a system of coated micropinocytotic vesicles. These vesicles avoided fusion with lysosomes by virtue of their protein coat, and discharged their contents at the basolateral membrane. Wild was unable to demonstrate that these coated

* However, subsequent studies demonstrated that human, rabbit and bovine IgG - HRP conjugates, rabbit anti-HRP antibodies, free HRP and human IgG all could be localised in coated micropinocytotic vesicles after exposure of the rabbit yolk-sac splanchnopleur to these proteins *in utero* (Moxon *et al* 1976).

vesicles contained endocytosed proteins. *

Recently, Rodewald (1980b) has reported that on exposing intestinal segments simultaneously to ferritin - IgG conjugates and free horse-radish peroxidase (HRP), both tracers enter the cell in the same endocytic vesicle. Only ferritin-IgG conjugates bind to the vesicle membrane, and are transferred to the abluminal surface. HRP remains in the apical cytoplasm and is concentrated in lysosomes. Thus the fluid contents of the endocytic vesicles do not follow the same pathway as the membrane bound contents. These recent findings suggest that uptake from the lumen may be a non-selective process, and that the segregation of molecules destined for digestion and those destined for transmission is an intracellular event; an observation which is contrary to his earlier studies.

The biochemical evidence concerning the level at which selection operates is equally as conflicting as the morphological evidence. Hemmings (1957) examined the fates of ¹³¹I labelled bovine and rabbit IgG injected into the uterine lumen of pregnant rabbits. The fetuses removed the same amount of bovine as rabbit IgG, thus absorption by the yolk-sac endoderm was non-selective. However, only a small proportion of the absorbed protein was recoverable intact in the foetus, the proportion of which depended upon the species of origin of the IgG. Since the fetuses themselves could not discriminate between bovine and rabbit IgG in their circulation (Hemmings and Oakley 1957), the conclusion was that selection occurred after entry of the IgGs into the yolk-sac splanchnopleur, but before their appearance in the foetal circulation.

Hemmings and Williams (1974) prepared nuclear, mitochondrial and microsomal fractions from rabbit yolk-sac endoderm and rabbit chorio-allantoic placenta, and examined the attachment of labelled rabbit and bovine IgG *in vivo* and *in vitro*. They also prepared similar fractions

from suckling rat gut and examined the binding of rat and sheep IgG. In all cases the attachment of homologous and heterologous IgGs was equal. Hemmings suggested that receptor binding could not account for selection, and stated that it may be the result of differential action by the lysosomal Cathepsins.

Jones (1976a) examined the uptake, by the suckling rat gut, of mixtures of homologous and heterologous IgGs labelled with ^{125}I and ^{131}I . For each protein pair examined, there was a higher concentration in the gut wall of the protein which was more efficiently transmitted intact to the vascular compartment. However there also appeared to be a preponderance of the less well transmitted proteins in the gut wash. Jones concluded from these findings that uptake from the gut lumen was selective. Examination of the $^{131}\text{I}/^{125}\text{I}$ ratios after the intraluminal injection of protein pairs also revealed that the preferential transmission of one IgG could not wholly be accounted for by its preferential removal from the gut lumen. Jones proposed differential catabolism for homologous and heterologous IgGs to explain this unaccounted for selection. The studies reported in this thesis, concerning the clearance of various IgGs from the plasma and their relative digestibility, shows this concept to be untenable. The results presented by Jones in support of selective uptake, probably indicated that IgG uptake from the lumen was preferential, but that some form of selection must also have operated intracellularly.

The results presented in Table 4.1 show that the proximal enterocytes transmitted intact IgG to the vascular compartment in the order rat>human>sheep>bovine. Ultrafiltration analysis of the serum, viscera and carcass homogenates revealed that these heterologous IgGs are processed in the same way as rat IgG; they are either transmitted intact or broken down to fragments smaller than 1000 mol.wt.

A possible explanation of why different amounts of IgG were trans-

mitted intact could be that, despite their affinity for the receptor, they were digested to differing extents in the gut lumen. The results show that this is unlikely since the IgGs rank in the following order with respect to digestibility; rat, sheep, bovine, human (Table 4.4) whereas the ranking order for transmission was rat, human, sheep, bovine.

Relative digestibility may be a minor factor in determining the number of intact molecules available for transmission, although even this is unlikely since as shown by the trypsin inhibitor studies mentioned previously, luminal digestion in the proximal small intestine was not significant.

Table 4.1 shows that there was no significant difference between the amount of radioactivity remaining in the gut lumen two hours after the intraluminal injection of rat, human, sheep or bovine IgG. However, there was significantly more radioactive material in the gut wall of the rat and human groups than in the bovine and sheep groups. Thus the initial uptake of IgG from the intestinal lumen was non-selective and selection occurred intracellularly. The fact that the percentage of a dose of IgG removed from the gut in two hours was independent of dose concentration also strongly suggests that uptake is a non-selective process (see Table 6.2).

The studies on the removal rates of various proteins from the vascular compartment (Fig. 4.10) showed that the heterologous IgGs were cleared at the same rate as rat IgG. Since the proximal small intestine removed the same amount of heterologous and homologous IgG from the gut lumen after two hours, and the IgGs were cleared from the plasma at the same rate, then the different amounts of intact IgG present in the vascular compartment must have been due to selection occurring inside the enterocytes. As shown by the kinetic studies on IgG transmission (Chapter 6), the digestive pathway was more rapid than the receptor mediated "protected" pathway. Thus the better the fit between IgG and receptor, the more

molecules will be processed by the protected pathway, and consequently the rate of throughput of radioactive material by the enterocytes will be slower. This explains why there was more radioactivity remaining in the gut wall after the intraluminal injection of rat and human IgG than sheep and bovine IgG.

Kinetic studies

The Brambell hypothesis (1970) made several predictions concerning the transmission of saturating and sub-saturating concentrations of IgG, and in order to test them it was necessary to quantify the saturability of the uptake mechanism in the system examined in this thesis. Table 6.1 and Fig. 6.1 show that a dose of between 500-750 μ g of rat IgG in 0.2 ml was sufficient to saturate the capacity of the ligated proximal small intestine of the 15 day old rat to transport intact IgG to the circulation.

Two conclusions were drawn from these saturation studies which were of relevance to the Brambell hypothesis. As predicted by Brambell, as the concentration of the administered dose of IgG was increased, so the proportion of the dose removed from the gut which was transmitted to the vascular compartment as intact IgG decreased. Conversely, as the dose concentration was increased, so the proportion of the dose removed which was broken down increased (See Table 6.2 and Fig. 6.2). These findings confirmed the results of Hemmings (1975a) and I. G. Morris (1976), although Hemmings subsequently claimed to be unable to demonstrate this phenomenon (1976). The results obtained were consistent with a saturable IgG receptor system.

Having established the level of IgG necessary to saturate the receptor population of the proximal small intestine, the patterns of rat IgG transmission at both saturating and sub-saturating concentrations

were examined over a six hour period.

The results in Fig. 6.4 revealed that the enterocytes loaded up with radioactivity very rapidly and endocytosis had ceased thirty minutes after injection. It is possible that the surface area of plasma membrane available for internalisation became limiting, due to the phase of rapid pinocytosis, and a period for membrane recycling was required before endocytosis could be resumed.

Having absorbed a proportion of the injected dose non-selectively, as explained previously, the enterocytes processed this radioactive material. At a saturating IgG concentration this processing resulted in a very rapid throughput of 300 µg of protein in the first thirty minutes after injection, almost all of which appeared as breakdown products in the viscera and carcass. This represented the proportion of the endocytosed dose of IgG which was not bound to receptors, and was consequently digested. Once the IgG has been digested, the fragments rapidly diffuse out of the enterocytes, leaving receptor bound IgG in the cells.

At an IgG concentration below saturation (Fig. 6.4), there was very little digestion of IgG, and most of the radioactivity removed from the gut appeared as TCA-precipitable material in the vascular compartment.

Three and a half hours after injection of the saturating IgG dose, the level of intact IgG in the vascular compartment reached a maximum. At this point a further increase in the radioactivity in the viscera and carcass was detected. This was probably due to a concentration-catabolism effect as described previously (see Chapter 6).

By means of *in vitro* studies on the binding of IgG by isolated jejunal enterocytes, Mackenzie (1981) estimated that there were $4.84 \pm 0.15 \times 10^6$ Fc receptors per enterocyte and 30×10^6 enterocytes in the proximal small intestine. Thus a total of 1.45×10^{14} receptor sites were estimated to be present in this region of the gut. From the results presented in table 6:3 it can be calculated

that the proximal small intestine was capable of transmitting to the vascular compartment 136.9 μg of IgG per hour. However, as can be seen from table 4:3, only 83.2% of this material represented IgG molecules with a molecular weight greater than 100,000. Thus the rate of transmission of intact IgG was:

$$\begin{aligned} & (136.9 \mu\text{g} \times 0.832) \text{ per hour} \\ & = 113.9 \mu\text{g per hour.} \end{aligned}$$

Using TCA precipitation as an estimate for intact IgG transmission, the maximum hourly rate of transmission becomes 135.0 μg . However, this is likely to be an overestimate due to the precipitation of some large IgG breakdown products.

Assuming a relationship of one IgG molecule binding to one receptor molecule, it is possible to calculate the receptor recycling time as follows:

$$\begin{aligned} 113.9 \mu\text{g of IgG} &= 7.59 \times 10^{-10} \text{ moles of IgG} \\ 7.59 \times 10^{-10} \text{ moles} &= (7.59 \times 10^{-10}) \times (6 \times 10^{23}) \text{ molecules} \\ &= 4.55 \times 10^{14} \text{ molecules.} \end{aligned}$$

Therefore 4.55×10^{14} molecules of IgG are transmitted per hour. This would theoretically involve the use of each of the 1.45×10^{14} receptors 3.14 times during the hour, or once every 19.1 minutes. Using the intact IgG transmission rate estimated by TCA precipitation, the receptor recycling time can be calculated to be 16.1 minutes.

Using *in vivo* techniques similar to those used in this thesis, Mackenzie (1981) estimated the maximum rate of intact IgG transmission by the proximal small intestine of 12 day old rats to be 157.34 μg per hour. To account for this rate of transmission, each of the available 1.45×10^{14} receptors in this region of the gut must be used 4.17 times per hour, or once every 14 minutes.

These estimates have certain implications on the IgG transport process.

The kinetic studies (Chapter 6) demonstrated that when proximal enterocytes were exposed to a dose of IgG, a certain proportion of the dose was rapidly endocytosed. After this initial phase, endocytosis appeared to cease. If one accepts a receptor recycling time of between 14-19 minutes, then it is unlikely that the limiting factor in the endocytic process would be receptor unavailability. In which case, it is more probable that the availability of plasma membrane for internalisation is the limiting factor.

The receptor recycling system is probably far more complex than these elementary attempts at quantification would imply, and further studies in this area are needed if a more precise understanding is to be achieved.

Subcellular fractionation

Morphological studies have been very useful in furthering our understanding of the IgG transport mechanism, however they can only give us a static picture of what is a dynamic process. Prediction of the mechanisms involved based on such information alone is very difficult.

One of the major problems regarding the claim that coated vesicles are involved in the transcellular transmission of intact IgG has been the failure to isolate these organelles, and to demonstrate that they contain intact IgG. Some of the difficulties in isolating coated vesicles from the neonatal gut are due to the relatively small quantity of tissue available. Initial attempts were made using the method of Pearse (1975) for the isolation of coated vesicles from porcine brain. However, using this technique the amount of material remaining after only a few centrifugation steps was too small for subsequent processing. An alternative procedure was developed which produced a crude microsomal pellet, which was fractionated by means of ultracentrifugation on a sucrose density gradient. This method was sensitive enough to detect radioactive material

associated with particles having a sedimentation coefficient of 250 S. This was very close to the value quoted by Crowther *et al* (1975) for coated vesicles from porcine brain. This material was examined under the electron microscope using negative staining. Polyhedral particles were present which had a diameter of about 70 nm - similar to the expected shape and size of coated vesicles. The quality of the micrographs was such that the lattice-work coat, which has been demonstrated in coated vesicles from pig brain and several other tissues, was not discernable.

Further evidence which suggested that these particles were coated vesicles was obtained from the SDS polyacrylamide gel electrophoresis of the crude coated vesicle pellet. This revealed a faint band of protein with a molecular weight of 180,000 - similar to that of the protein "Clathrin", which is considered to be characteristic of coated vesicles.

As demonstrated by kinetic studies (Fig. 6.3) and by the gel filtration analysis of the gut wall contents (Fig. 5.1) one hour after the injection of labelled IgG into the proximal small intestine almost all of the IgG contained and transmitted by the enterocytes was intact. Thus the observation that radioactive material was associated with 250 S particles, one hour after the intraluminal injection of labelled IgG, strongly supports the involvement of coated vesicles in the intact IgG transport process.

It was necessary to attempt to purify the lysosome population of the proximal enterocytes to examine one of the most fundamental aspects of the transport mechanism - namely, does intact IgG destined for trans-cellular transmission enter phagolysosomes as predicted by the Brambell hypothesis? A technique was developed which produced a fraction with a concentration of lysosomal marker enzymes of up to five times higher than that of the original homogenate. However, this could not be described as a pure lysosomal preparation since it may have been contaminated

with organelles for which enzyme markers are not known or where not used.

Analysis of the radioactivity associated with this preparation (see Fig. 7.6) indicated that two minutes after the intraluminal injection of labelled IgG, both intact IgG and breakdown products were present.

However, after half an hour only intact IgG could be detected. This may mean that IgG destined for transmission does pass through the lysosomal system as predicted by Brambell, however it does not confirm this aspect of his hypothesis due to the reservations mentioned previously.

The mechanism of transcellular IgG transport

Mechanisms which are proposed to describe the transcellular transport of IgG must account for the following observations:

- a) Selection, operating not only between very different molecules such as albumin and IgG but also between very similar ones such as rat and bovine IgG.
- b) Interference by one IgG with the transmission of another.
- c) Both selection and interference being associated with the Fc terminal.
- d) The saturability of the transmission of intact IgG. Excess IgG being digested by the cell.
- e) Uptake of IgG from the intestinal lumen by the enterocytes being a non-selective process.
- f) The involvement of coated vesicles in the transmission of IgG.
- g) The possibility that IgG destined for transmission may pass through lysosomes.

In recent studies, Rodewald (1980b) observed that after exposure of segments of the proximal small intestine to ferritin-IgG conjugates (Ft-IgG) and horseradish peroxidase (HRP), both tracers entered the cells

within the same endocytic vesicles at the luminal surface. However only the Ft-IgG bound to the vesicle membrane, was transferred to the abluminal membrane and was released from the cells. HRP remained in the apical cytoplasm where it was concentrated within lysosomes. Thus endocytosis was a non-selective event, and the vesicle contents were sorted within the enterocytes.

These findings lend support to the observation reported in this thesis - that the uptake of IgG by the proximal small intestine was non-selective. However, these findings are basically different from the earlier suggestions made by Rodewald (1973) and Wild (1976), who claimed that protein destined for transcellular transport was selectively endocytosed at the apical cell surface.

The intracellular sorting process presumably involves the transfer of receptor-bound IgG from the endocytic tubular vesicles to coated vesicles, which subsequently carry the IgG across the cell and discharge it at the baso-lateral membrane with the aid of a pH shift. The non-receptor-bound material remains in the tubular vesicle and is degraded when the vesicle fuses with a lysosome (see Fig. 8.1). However the results of the lysosome isolation experiments do not exclude the possibility that the endocytic vesicles fuse with lysosomes prior to the transfer of receptor-bound IgG to coated vesicles (see Fig. 8.2), although there is no morphological evidence in support of such a mechanism.

FIG. 8.1

Diagrammatic representation of IgG transport across enterocytes from the proximal small intestine of the neonatal rat (1).

Both receptor-bound and non-receptor-bound IgG internalised by specialised pits located on the apical cell membrane.

(2) Receptor-bound and non-receptor-bound IgG located in small tubular vesicles. (3) Receptor-bound IgG transferred from the tubular vesicles to coated vesicles, which travel through the enterocyte and discharge the IgG at the basolateral membrane by reverse pinocytosis. (4) Tubular vesicles, containing only non-receptor-bound material fuse with lysosomes (L), and the vesicle contents are digested.

FIG. 8.2

Diagrammatic representation of IgG transport across enterocytes from the proximal small intestine of the neonatal rat.

(1) Both receptor-bound and non-receptor-bound IgG internalised by specialised pits located on the apical cell membrane.

(2) Receptor-bound and non-receptor-bound IgG located in small tubular vesicles. (3) Fusion of tubular vesicles with lysosomes (L). Non-receptor-bound material is digested.

(4) Receptor-bound IgG is protected from lysosomal enzymes, and transferred to coated vesicles which travel through the cell and discharge the IgG at the basolateral cell membrane by reverse pinocytosis.

FIG. 8.1

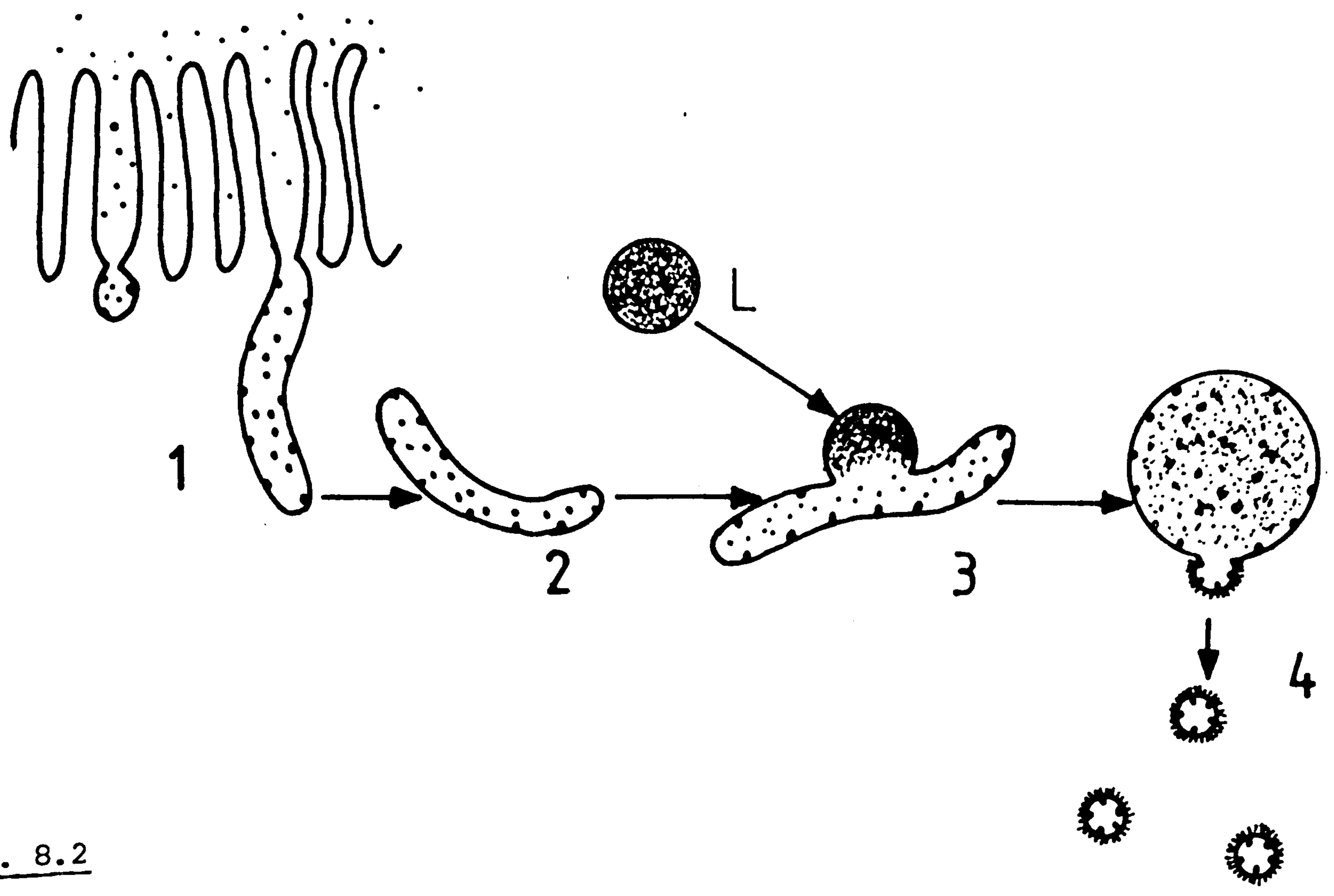
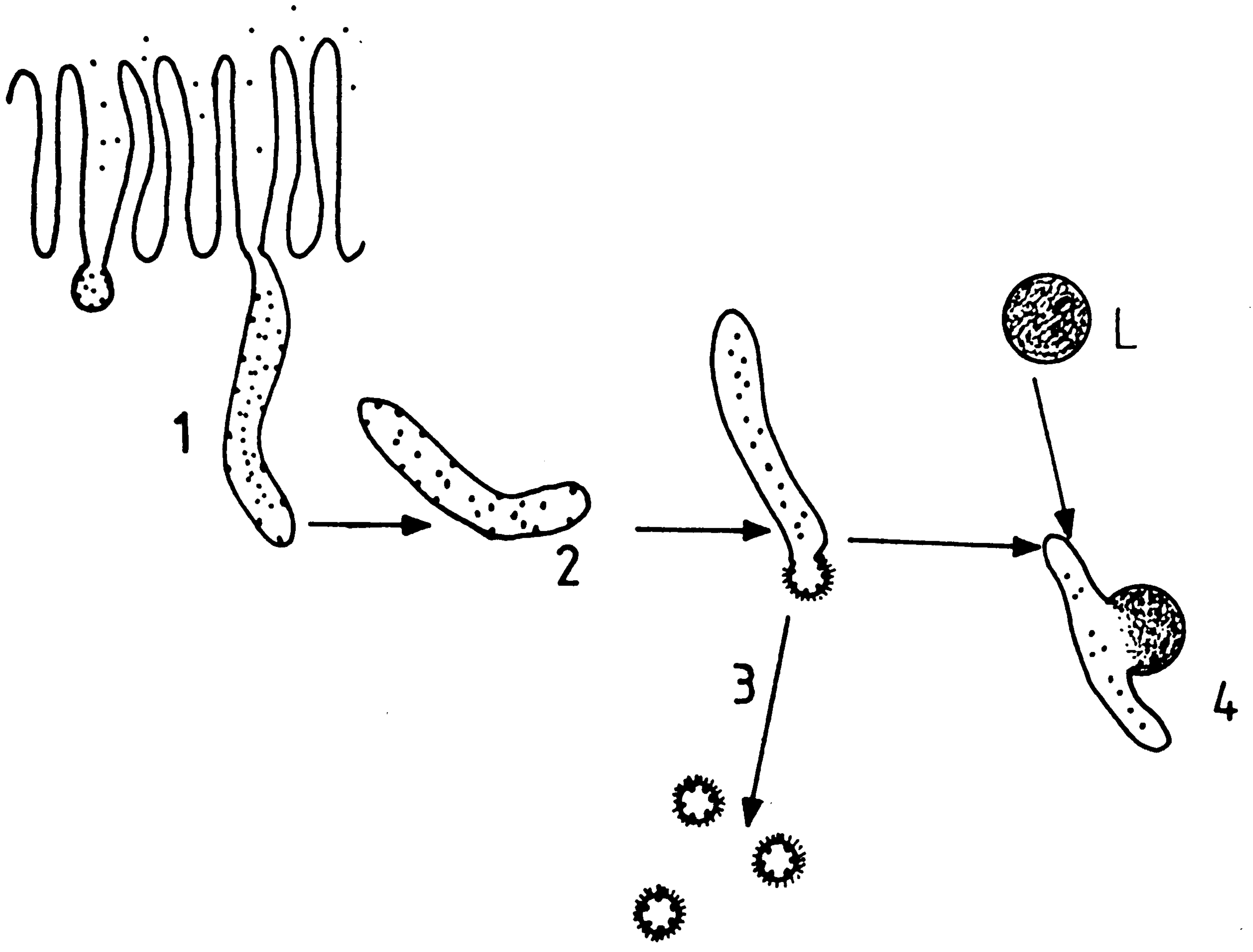


FIG. 8.2

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