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The Transmissions of Antibodies Across the Gut of Pouch-Young Marsupials

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Summary. The transmission of antibodies across the gut of suckling pouch-young was investigated in three species of marsupials (*Setonix brachyurus*, *Macropus eugenii* and *Trichosurus vulpecula*) from Australia.

Mother *Setonix*, immunized against *Salmonella adelaide* flagella and Bacteriophage $\Phi \times 174$, transmitted the antibodies in milk to their young. In sucrose density gradient runs, the antibody activity in milk whey and in serum of pouch-young, of *Setonix* and *Macropus* was found to be in the 7S region only; antibody in the 11S and 19S regions was not detected. Chromatographic preparations of IgM antibodies were fed to pouch-young *Setonix* which were later bled and their serum titrated for anti-*S. adelaide* agglutinins and antiphage $\Phi \times 174$ activity. The IgM antibodies were not transmitted across the gut in detectable amounts.

Antibodies were present in the blood of pouch-young *Setonix* within 15–60 minutes of gavage (feeding by stomach tube) of immune serum. In *Setonix* the capacity to absorb antibodies in the intestine was lost at an age between 170 and 200 days and in *Trichosurus* it was lost at an age between 98 and 145 days. At these ages the pouch-young were able to leave the marsupium for varying lengths of time. Antibodies did not traverse the rumen wall in a young *Setonix* whose rumen was isolated from the intestine with ligatures before immune serum was gavaged.

INTRODUCTION

Marsupials have a special attraction for biologists chiefly because (a) they are a phyletic line separate from eutherians (Simpson, 1945) and they provide useful comparative analogues (Waring, Moir and Tyndale-Biscoe, 1966), and (b) their young are born at a state equivalent in general development to a eutherian foetus which is immunologically incompetent (Block, 1964; Yadav and Papadimitriou, 1969). The newborn climbs into the pouch, attaches to a teat and undergoes further development in conditions very unlike those in the uterus. Before our investigations there has been little interest in the immune mechanisms that help young marsupials to survive in the pouch environment. It is very likely, as in eutherians, that maternal antibody provides the initial immune protection to the young. Passage of maternal antibodies from mother to young may occur prenatally, or postnatally or as a combination of the two (Brambell, 1958). Whereas in ungulates all

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maternal immunoglobulins are derived after birth from the colostrum and milk, in rodents (Halliday, 1955) the hedgehog (Morris, 1961) and the dog (Mason, Dalling and Gordon, 1930) maternal immunoglobulins are transmitted both before and after birth; generally, more immunoglobulins are passed by way of the milk than across the placenta. The duration of the capacity of the gut to absorb immunoglobulins varies in different species. It lasts for up to 24–36 hours in ungulates (Leece and Morgan, 1962; Kim, Bradley and Watson, 1966), for 16 days in the mouse, 20 days in the rat (Halliday, 1955), and for 40 days in the hedgehog (Morris, 1961).

We have reported previously (Yadav, Eadie and Stanley, 1971) that there were no immunoglobulins detectable in the blood of prenatal and newborn quokka (*Setonix brachyurus*), a diprotodont marsupial indigenous to Western Australia, before it has suckled. The transmission of immunoglobulins from mother to young occurred with the ingestion of colostrum, and the ability to absorb antibodies from the milk persisted for 6 months up to the time when the young left the pouch.

The experiments described here were designed to confirm the earlier work in the quokka and extend the observations to two other Australian marsupials. In this study some observations are made on the class of immunoglobulins transmitted in the marsupial.

MATERIALS AND METHODS

Animals

Suckling pouch-young of three species of marsupials were used; the quokka (*Setonix brachyurus*), the tammar (*Macropus eugenii*) and the possum (*Trichosurus vulpecula*) were obtained from Rottnest Island, Garden Island and Perth in Western Australia, respectively. The quokkas and tammars were held in outdoor pens and the possums were kept in large outdoor cages. Food and water were given *ad libitum*. Table 1 summarizes the data on these animals relevant to this paper. After vacating the pouch the young suckle from outside the pouch for varying lengths of time.

TABLE 1

	Quokka	Tammar	Possum
Gestation (days)	27	29	17
Pouch term (days)	180	250	120
Litter size	1	1	1
Age weaned (days)	~400	~400	~250
Mean weight of Newborn (mg)	330	450	200
Mean weight of adult female (kg)	2·4	3·9	2·2
Type of digestion	ruminant	ruminant	non-ruminant

Preparation of immune sera

Healthy adult animals were immunized with sheep erythrocytes (2 ml of 20 per cent suspension, i.p.), bacteriophage $\Phi \times 174$ (1 ml of 10^9 plaque-forming units, i.p.) and *Salmonella adelaide* flagella (0·5 ml of 1 mg/ml solution, i.m.). Using the same doses the animals were injected twice more at 3-week intervals, before being bled 7 days after the last injection. An aliquot of the serum was used for gavage trials and the rest for isolation of 19S and 7S antibodies.

Gel filtration of the marsupial immune serum on Sephadex G-200 columns (2·5 cm × 185 cm) by the upward flow method using 0·1 M Tris-HCl buffer containing 0·1 M NaCl (pH 8·0) gave three protein peaks. Fractions eluted from the leading half of the first peak were pooled and concentrated by negative pressure dialysis. Antibody activity was present in this preparation and immunoelectrophoresis showed four protein arcs, three of which were identified as 19S immunoglobulin, α_2-macroglobulin and β-lipoprotein; no IgG immunoglobulin was detected in the first peak. The preparation was used as a source of 19S antibody.

IgG immunoglobulin was made by passing 40 ml of the immune serum through a DEAE-cellulose column (2 × 25 cm). The protein eluted with 0·005 M phosphate buffer (pH 7·0) was predominantly IgG. Antisera to quokka IgG immunoglobulin and to quokka serum was made in rabbits.

Sucrose density gradient (SDG) ultracentrifugation

A 1 ml sample of serum and of milk whey was diluted 2:3 with physiological saline and layered on to a 4 ml linear gradient of 10 per cent to 40 per cent sucrose in 0·15 M NaCl. The gradients were then centrifuged for 18 hours at 35,000 rev/min using a swinging bucket rotor (SW 25·2) in a Spinco model L ultracentrifuge. Eluates (0·23 ml volume) were collected from the base of the cellulose centrifuge tubes with the aid of a fraction collector monitored for drop-counting, and from the fractions collected, 50 μl samples were removed for antibody assay before protein determination. To each SDG fraction 3 ml of 0·85 per cent saline was added and the protein estimated at 280 mμ in a spectrophotometer.

The class of the immunoglobulins present in the SDG fractions was determined by agar-gel precipitin reactions using appropriate rabbit antisera. Human IgA (11S) was added as marker in duplicate sucrose density runs of tammar milk whey and was identified in the fractions by immunoelectrophoresis using specific antihuman IgA serum, prepared in rabbits. Dr K. J. Turner kindly supplied the human IgA immunoglobulin and antihuman IgA serum.

Agar diffusion

Immunoelectrophoresis was carried out by Scheidegger's (1955) method. Two and a-half millilitres of 1 per cent Oxoid Ionagar No. 2 in 0·1 M sodium barbital buffer (pH 8·6) were layered on microscope slides. After charging the wells with antigen each slide was subjected to 50 V for 1 hour. The troughs were then filled with appropriate antiserum after which the slides were incubated at 23° for 12–24 hours. At the end of the time the slides were dried in an incubator (60°) and the dried slides were stained with azocarmine.

Gavage of immune fractions to pouch-young

A stomach tube was prepared by attaching a 5 ml plastic syringe to an intracatheter. The pouch-young was gently eased off the teat and without anaesthesia the stomach tube was passed down into the rumen. The desired volume of immune serum or fraction was deposited in the rumen and if this was done slowly and carefully there was no regurgitation of the sample. For technical reasons pouch-young less than 40 days were not used. In pouch-young older than 180 days a rubber stomach tube was used; the animals were anaesthetized before gavage.

Antibody assay

The antibodies were titrated by the microcapillary method. Agglutinins to *Salmonella adelaide* were assayed by incubating at 37° for 1 hour, the appropriate dilutions of serum in saline, with equal volumes of formalin killed suspension of *S. adelaide* bacteria. The tubes were read at ×5 magnification for distinct agglutination.

Neutralizing activity to bacteriophage Φ × 174 was estimated using the method of Adams (1959). Standard volumes of serum and phage Φ × 174 were incubated at 37° for 60 minutes. The viable phage present in the mixture were counted and the K value computed.

Haemolytic antibody to sheep red blood cells (SRBC) was titrated by the microtechnique routinely used in this department. The sera were not inactivated because preliminary tests showed that part of the haemolytic antibody formed in the marsupials was thermolabile. Equal volumes of a 3 per cent suspension of SRBC containing a standard amount of complement (fresh guineapig serum) and the appropriately diluted serum were mixed in microcapillary tubes prior to incubation in a water bath at 37° for 30 minutes. Positive results were recorded for those tubes showing 50 per cent to 100 per cent haemolysis.

RESULTS

TRANSMISSION OF PASSIVE IMMUNITY TO SUCKLING POUCH-YOUNG

Figs. 1 and 2 illustrate the antibody levels to *Salmonella adelaide* and phage Φ × 174 respectively, of an adult female and its suckling pouch-young at different times after the immunization of the mother. The anti-*S. adelaide* titres in the pouch-young closely follow the levels present in the mother (Fig. 1) but the antiphage Φ × 174 titres are much lower in the pouch-young than in the mother (Fig. 2). The simplest explanation for this difference in the transmission of two separate antibodies in the same animal is that the antibodies occur in different immunoglobulin fractions and not all fractions reach the circulation of the pouch-young.

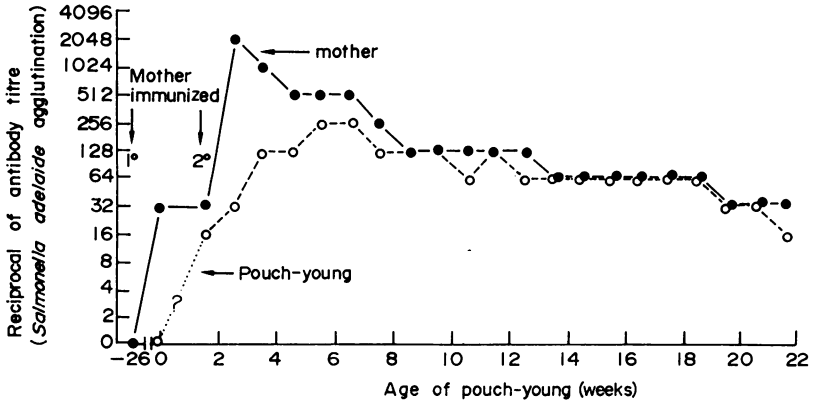

FIG. 1. The agglutinin titre of mother (No. 6533) (●) and suckling pouch-young (○) *Setonix brachyurus*. The mother was immunized with *S. adelaide* (1 mg in 1 ml i.m.) 26 weeks before and 1 week after the birth of the young.

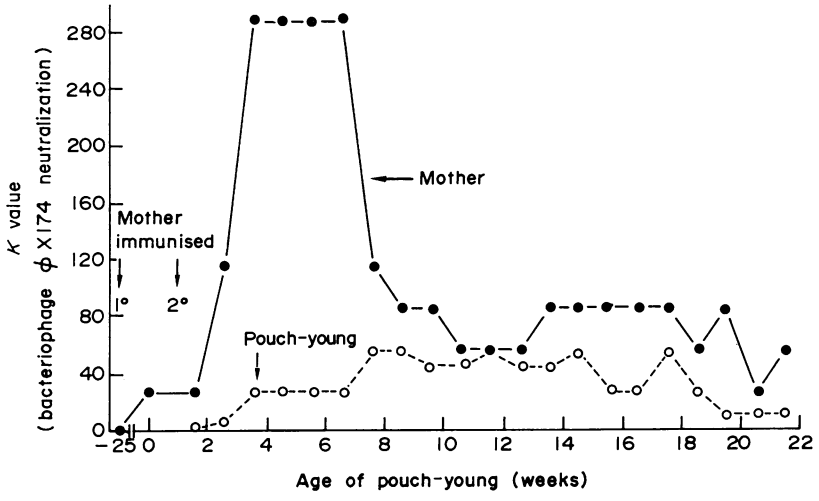

FIG. 2. Antiphage titres of mother (No. 6533) (●) and suckling pouch-young (○) *Setonix brachyurus*. The mother was immunized with bacteriophage Φ × 174 (6×10^8 pfu i.p.) 26 weeks before and 1 week after the birth of the young.

RATE OF INTESTINAL UPTAKE OF SERUM ANTIBODIES

Antibodies were present in the blood of pouch-young quokka aged 95 days within 15–60 minutes of gavage of immune serum (Table 2). The peak antibody titre to *S. adelaide* flagella and phage Φ × 174 was reached in 2 and 4 hours respectively. In the next 24 hours the antiphage Φ × 174 titre dropped but anti-*S. adelaide* titre remained at a constant level. Fifty-five days after ingestion of immune serum, anti-*S. adelaide* agglutinin was not detected but a trace amount of antiphage Φ × 174 activity was present in the blood of the pouch-young.

TABLE 2

THE APPEARANCE OF ANTIBODY IN SERUM OF POUCH-YOUNG AGED 95 DAYS (WEIGHT 61·2 G, SEX: FEMALE) FED 0·5 ML OF IMMUNE SERUM CONTAINING ANTI-*S. adelaide* AGGLUTININS (TITRE 1/8192) AND ANTIPHAGE Φ × 174 ANTIBODIES ($K = 2882$)

Time from feeding	Antibody in serum of pouch-young	
	Antiphage Φ × 174 (*K* value)	Anti-*S. adelaide* agglutinins (reciprocal of titre)
0 minutes	0	<2
15 minutes	0·05	4
60 minutes	0·58	8
2 hours	8·65	32
4 hours	28·82	32
8 hours	11·53	32
12 hours	8·65	32
24 hours	5·76	32
7 days	2·88	16
28 days	2·88	8
55 days	0·05	<2

TABLE 3

THE INABILITY TO ABSORB ANTIBODIES FROM THE IMMUNE SERUM IN RUMEN OF POUCH-YOUNG AGED 95 DAYS (WEIGHT 65 G, SEX: FEMALE)

Serum samples tested	Antibody titre		
	SRBC hemolysins (reciprocal of titre)	Anti-*S. adelaide* agglutinins (reciprocal of titre)	Antiphage Φ × 174 (*K* value)
Hyperimmune sera fed to young	64	65536	865
Pouch-young			
Bled before ingestion	<2	<2	0
Bled 4 hours after ingestion	<2	2	0
Bled 24 hours after ingestion	<2	2	0

SITE OF ANTIBODY ABSORPTION IN POUCH-YOUNG

In a quokka pouch-young aged 95 days the rumen was isolated from the duodenum with two ligatures using fine silk thread before immune serum was fed by a stomach tube. The operation was performed in order to determine whether immunoglobulins traversed the rumen wall. The pouch-young reattached to the teat on being returned to the pouch. Table 3 shows that antibody was not detected in the blood of the pouch-young even after 24 hours. This observation suggests that the rumen wall in pouch-young is impervious to immunoglobulins.

Autopsy examination of the pouch-young revealed that the stomach contained a milky fluid. The stomach was greatly distended from fermentation gases normally formed there and by contrast the intestine was empty.

SELECTIVE ABSORPTION OF IMMUNOGLOBULINS

Table 4 shows that 19S antibodies were not transmitted across the gut of young quokkas in detectable amounts but 7S antibodies passed readily.

TABLE 4

THE INABILITY OF POUCH-YOUNG QUOKKA TO ABSORB IgM IMMUNOGLOBULINS IN THE GUT

Quokka pouch young (ml gavaged)	Age (days)	Antibody titre					
		SRBC haemolysins (reciprocal of titre)		Anti-*S. adelaide* agglutinins (reciprocal of titre)		Antiphage Φ × 174 (*K* value)	
Quokka IgM fed to young	—	128		512		3	
		Pre-bleed	Post-bleed	Pre-bleed	Post-bleed	Pre-bleed	Post-bleed
No. 2919, wt 33 g (gavaged 1 ml)	75	<2	<2	<2	2	0	0
No. 2918, wt 53 g (gavaged 2 ml)	90	<2	<2	<2	2	0	0
Quokka IgG fed to young	—	128		2048		58	
		Pre-bleed	Post-bleed	Pre-bleed	Post-bleed	Pre-bleed	Post-bleed
No. 2930, wt 53 g (gavaged 2 ml)	90	<2	8	<2	32	0	5·76

TABLE 5

THE CAPACITY OF POUCH-YOUNG MARSUPIALS TO ABSORB ANTIBODY FROM IMMUNE SERA IN THE ALIMENTARY TRACT

Species	No.	Sex	Age (days)	Millilitres of immune serum fed	Source of immune serum*	Antibody titres—reciprocal of titres							
						SRBC haemolysis		SRBC agglutinins		*S. adelaide* agglutinins		*antiphage* Φ × *174* (*K* value)	
						Pre-bleed	Post-bleed	Pre-bleed	Post-bleed	Pre-bleed	Post-bleed	Pre-bleed	Post-bleed
Setonix brachyurus	2980	♂	270	6	*Setonix*[1a]	<2	<2	<2	<2	<2	<2	0	0
	2951	♀	233	3	*Setonix*[1a]	<2	<2	<2	<2	<2	<2	0	0
	2936	♂	226	3	*Setonox*[1a]	<2	<2	<2	<2	<2	<2	0	0
	10C–1	♂	216	3	*Setonix*[1a]	<2	<2	<2	<2	<2	<2	0	0
	10C–2	♀	201	3	*Setonix*[1a]	<2	<2	<2	<2	<2	<2	0	0
	10C–3	♀	170	3	*Setonix*[1a]	<2	<2	<2	<2	<2	32	0	29
	6577	♀	95	0·5	*Setonix*[1a]	<2	<2	<2	<2	<2	32	0	29
Macropus eugenii	T–1	♀	60	1	*Trichosurus*[b]	<2	<2	ND	ND	<2	128	0	12
Trichosurus vulpecula	JP–1	♀	145	2·5	*Trichosurus*[b]	<2	<2	ND	ND	<2	<2	0	0
	JP–2	♂	98	1·5	*Trichosurus*[b]	<2	<2	ND	ND	<2	1024	0	9
	JP–3	♀	50	0·5	*Trichosurus*[b]	<2	32	ND	ND	<2	4096	0	288

* The antibody titres of the immune serums used in gavage were: (a) SRBC haemolysins 1/16; SRBC haemagglutinins 1/16; anti-*S. adelaide* agglutinins 1/256; and phage Φ × 174, *K*58. (b) SRBC haemolysin 1/1024; anti-*S. adelaide* agglutinins 1/65,536; and phage Φ × 174, *K* = 865.

ND, Not done.

TERMINATION OF THE CAPACITY OF THE GUT TO ABSORB ANTIBODY

Juvenile quokkas (older than 200 days) and possum aged 145 days were unable to absorb antibodies from the homologous immune serum gavaged (Table 5). However, the pouch-young quokkas aged less than 170 days and the possums aged 98 days or less were able to absorb the antibodies from the homologous immune serum. In quokka the ability to absorb antibodies from the intestine is lost at an age between 170 and 200 days; in

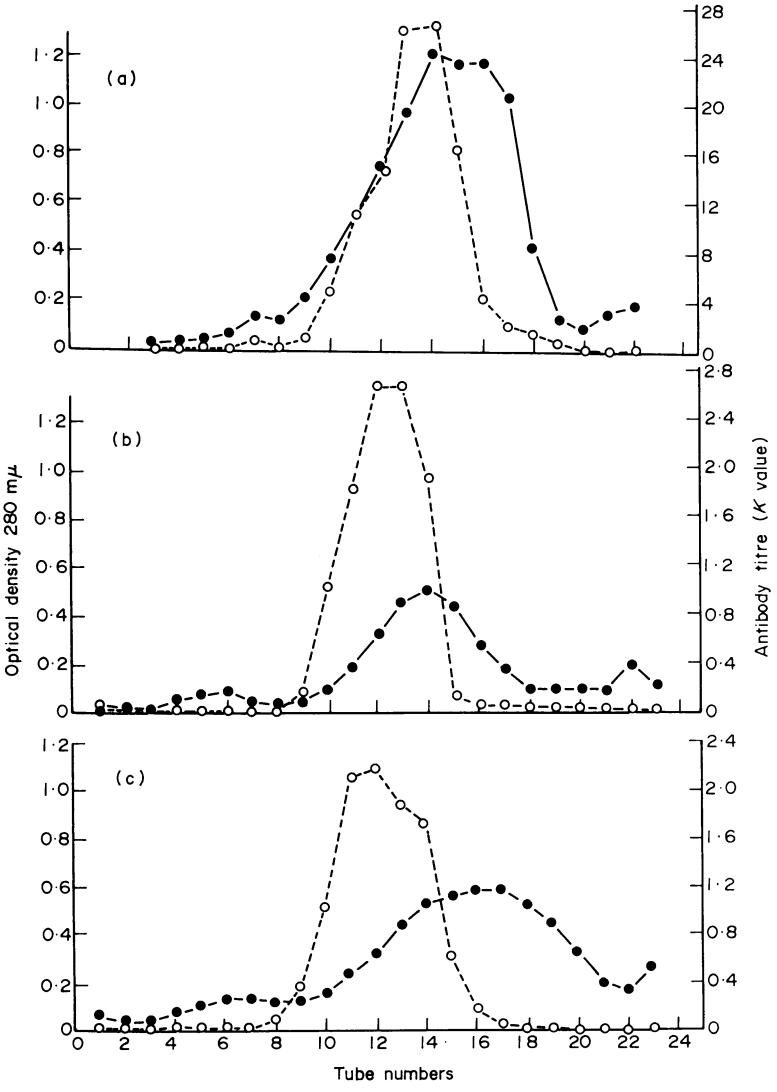

FIG. 3. Shows the antiphage Φ × 174 titres and sucrose density gradient protein profiles of *Setonix brachyurus* (a) in adult serum from immune mother, (b) in pouch-young serum after suckling and (c) in milk whey. ●, Optical density at 280 mμ; ○, antibody titre (*K* value).

possum at an age between 98 and 145 days. This confirms the previous observation in the quokka (Yadav, 1969) which showed that the capacity to absorb antibody was lost at about the time of leaving the pouch.

One further point from Table 5 needs emphasis. SRBC haemolytic antibody was transmitted in the possum-young aged 50 days but not in possum aged 98 days and this implies that pouch-young lose the ability to absorb various antibodies at different ages; this aspect needs further investigation.

Heterologous antibodies were readily transmitted in the tammar pouch-young aged 60 days.

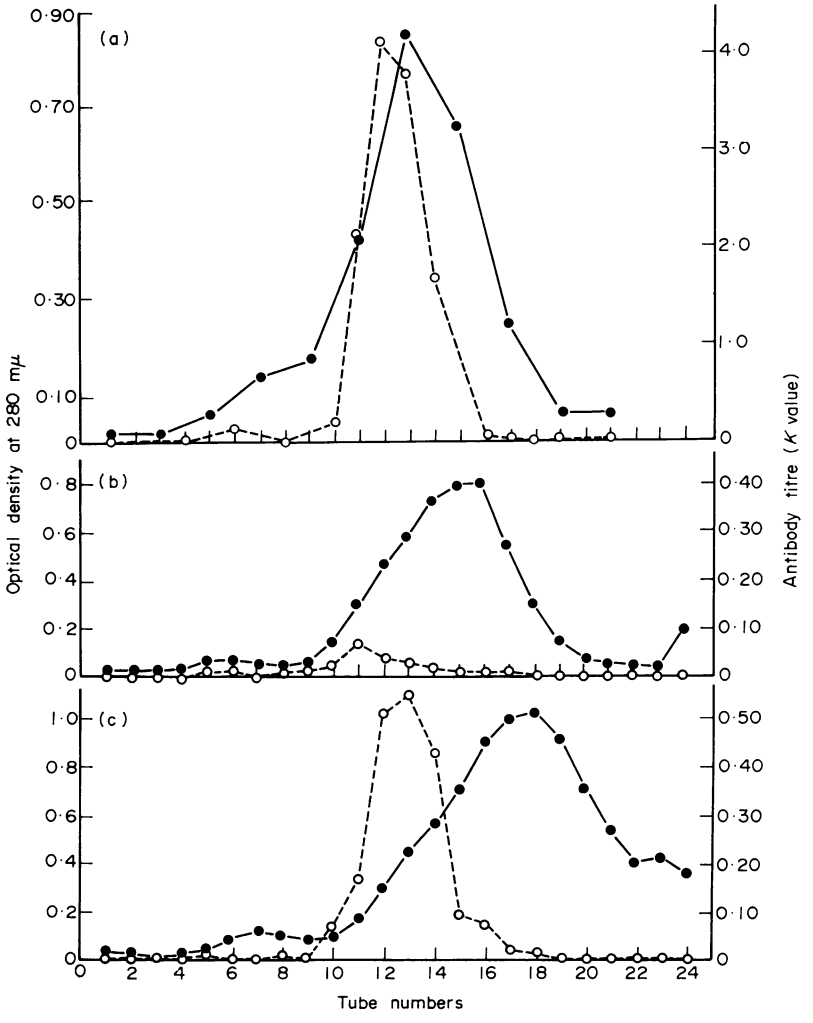

FIG. 4. Shows the antiphage Φ × 174 titres and sucrose density gradient protein profiles of *Macropus eugenii* (a) in adult serum from immune mother, (b) in juvenile serum after suckling and (c) in milk whey. ●, Optical density at 280 mμ; ○, antibody titre (*K* value).

ANTIBODIES IN MILK AND SERUM OF SUCKLING YOUNG

The protein sedimentation profiles on SDG of serum and milk are shown in Fig. 3(a–c) for quokka and in Fig. 4(a–c) for tammar. The distribution of antiphage Φ × 174 titres is superimposed on the protein concentration profile for each Figure. The results show that the antiphage Φ × 174 activity in the pouch-young quokka (Fig. 3c) or juvenile tammar serum (Fig. 4c) and the milk (Figs. 3b and 4b respectively) taken by the young has a very similar distribution with the median in the 7S region. The lack of a secondary antibody band in the 19S region could be due to low level of 19S antibody in the adult quokka and tammar immune serum.

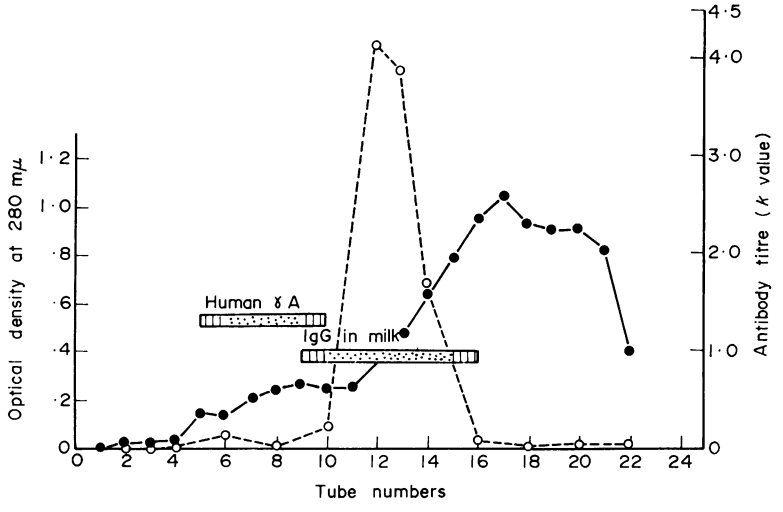

FIG. 5. Fractions from sucrose density gradient runs of *Macropus eugenii* immune milk (with purified human IgA added) showing localization of human IgA immunoglobulin and tammar IgG immunoglobulin detected by immunoelectrophoresis. Most of the antiphage Φ × 174 antibodies in the milk are restricted to the 7S region.

Fig. 5 shows the sedimentation profile of human IgA and tammar IgG in milk on sucrose gradient. A distinct antibody titre peak corresponding to the region of human IgA was not observed in the tammar milk. All antibody in milk was in the 7S region. A similar result was obtained for the quokka milk (see Fig. 3b).

DISCUSSION

In a previous communication (Yadav *et al.*, 1971) it was reported that the quokka derived all maternal immunoglobulins from colostrum and milk in the intestine. The passage of maternal antibodies across the gut wall occurred for 6 months which was approximately the time the young occupied the pouch. The work reported in this paper confirms the earlier observations for the quokka and in addition shows that intestinal absorption of immunoglobulin occurs in two other marsupials, the possum and the tammar. Like

the quokka, the possum loses its ability to absorb antibodies from the gut at the time it prepares to leave the pouch.

Antibodies appeared in the circulation of a young quokka within 15–60 minutes of gavage of homologous immune serum. Similar results were reported for the pig and the rat by Kraehenbul and Campiche (1969). The delay in absorption may be explained by the time taken for the immune serum or milk to reach the absorptive sites. In rats and mice, the chief site of absorption of immunoglobulins was the jejunum and ileum; antibodies did not readily cross the stomach wall or that of the duodenum (Halliday, 1956; Clark, 1959). In the hedgehog the stomach and duodenum appeared to be the chief sites of antibody absorption (Morris and Steel, 1967). Immunoglobulins did not traverse the wall of the rumen in the quokka and all transfer occured in the intestine. In marsupial pouch-young nearing the end of pouch-term, the intake of solid food and the absorption of antibodies from milk overlaps. Observations on freshly killed quokkas suggest that milk and other fluids by-pass the rumen and enter the intestine directly via the oesophageal groove. The method by which the milk is diverted to the absorptive sites in the intestine in the non-ruminant hedgehog (Morris and Steel, 1967) and the possum where immunoglobulin absorption continues to occur undiminished despite increased solid food intake is not known; most of the immunoglobulins would be destroyed by protein enzymes if the milk and ingesta mixed in the alimentary tract.

TABLE 6

PASSIVE IMMUNITY IN FOETAL AND NEONATAL MAMMALS

Species	Transfer through	Passage of immunoglobulins		Reference
		IgM	IgG	
Rat	Yolk sac and colostrum	–	γ_1 +, γ_2 +++	Morris (1967, 1969)
Human	Placenta only	–	+++	Smith (1960)
Pig	Colostrum only	+	+++	Locke *et al.* (1964) Porter (1969)
Calf	Colostrum only	+++	+++	Klaus *et al.* (1969)
Quokka	Colostrum only	–	+++	Yadav (1969)

The transmission of lgM immunoglobulins from mother to young occurs in some species only (Table 6). In quokka, lgM antibodies (19S rich Sephadex G-200 fraction) could not be shown to cross the gut wall in pouch-young older than 75 days. However, the possibility exists that the 19S antibody may be absorbed by the neonatal pouch-young but because of technical difficulties this could not be tested. The low levels of 19S antibody in immune milk suggests that they do not contribute significantly to passive immunity in the pouch-young. Rowlands and Dudley (1969) have reported the transmission of very small amounts of maternal antibody to bacteriophage f_2 to the nurseling pouch-young *Didelphis* aged 3–60 days. This observation suggests absence of transfer of IgM antibodies in *Didelphis* since in the mother of the pouch-young tested the IgM antibody titre in the circulation was higher than the IgG antibody.

Only one antibody peak was observed in the 7S region in SDG separation of immune milk from both the quokka and tammar; there was no secondary peak in the 11S region

of the SDG spectrum. At present we cannot say if this is due to the absence of 11S immunoglobulins in the milk or the absence of antibody activity in the 11S immunoglobulin. There is some evidence from the rat (Stechschulte and Austen, 1970) the pig (Porter, 1969) and the hamster (Bienonstock and Bloch, 1970) which suggests that colostral IgA is not absorbed but by remaining in the alimentary tract provides protection against bacteria and endotoxins (Porters, Noakes, and Allen, 1970). It appears that in marsupials 11S antibodies if present, are not prominently associated with protection in early life.

Marsupials develop the capacity to synthesis antibodies very early in life even though at birth they are relatively immature compared to eutherians. The newborn *Didelphis virginiana* (Block, 1964), *Setonix brachyurus* (Yadav and Papadimitriou, 1969), *Isoodon obsulus* and *Dasyurus viverrinus* (unpublished observation) do not have small lymphocytes in the thymus or elsewhere in the body but in the 4–6-day old *Didelphis* and *Setonix* small lymphocytes are present in the thymus, the cervical lymph node and the blood circulation. On day 8 and not before, *Didelphis* (Kalmutz 1962; La Via, Rowlands and Block, 1963; Rowlands, La Via and Block, 1964) and *Setonix* (Stanley, Waring and Yadav, 1966; Yadav, 1969) respond to injections of some antigens by formation of antibodies. It seems the pouch secretions which contain immunoglobulins and which appear to keep the pouch in the early days relatively free from bacteria (unpublished data) together with the milk immunoglobulins provide immune protection against infection to the young for up to 8 days. This paper presents no evidence as to the significance of the long duration of the capacity of the pouch-young to absorb maternal immunoglobulins from the gut but it appears likely that this capacity which occurs for longer than is found in any eutherian, is related to their precocious state of development at birth.

This study based on few animals gives only an introduction to the factors responsible for the immunological adaptation of the young to the marsupium and further investigations are clearly needed on these and other species for a critical appreciation of this interesting situation.

ACKNOWLEDGMENTS

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