QUANTITATIVE STUDIES ON TISSUE TRANSPLANTATION IMMUNITY

III. ACTIVELY ACQUIRED TOLERANCE

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CONTENTS

'Actively acquired tolerance' introduces into immunology the concept of a specific inhibition of response. Tolerance of a tissue homograft comes about when an animal has been confronted in foetal life with cells taken from its future donor, or from some other member of the donor's inbred strain (§3·1). It depends (*a*) upon an embryo's inability to respond to antigens by becoming immunized, and (*b*) upon its continued inability to do so in later life. Methods for inducing tolerance in mice (§§3·2, 4·1, 9), rats and rabbits (§3·4), and birds (§§3·3, 5, 7) are described in full.

In normal development, response to an antigenic stimulus by becoming tolerant gives way to response by becoming sensitized or immune. The transition from the one modality of response

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to the other occupies a 'null period' during which the exposure of animals to an antigenic stimulus has no appreciable effect. Most but not all mice and birds at birth or hatching have already entered this transitional period (§§4·1, 5).

Tolerance is antigenically specific. An animal injected in foetal life with cells from a donor *A* becomes completely tolerant of homografts transplanted in later life from a donor *B* if, and only if, *B* contains no antigens that are not also present in *A*. (In practice, this condition is most easily fulfilled when *A* and *B* are members of the same highly inbred strain.) The reaction of a tolerant animal against a homograft from an unrelated donor is not perceptibly impaired. Tolerance does not, however, discriminate between the tissues of a single individual; the inoculation of foetal or newborn mice with leucocytes or with the cells of a mammary carcinoma may confer tolerance of later homografts of skin (§§4·2, 9).

Tolerance of a homograft is neither caused by nor accompanied by an antigenic adaptation of the grafted cells. An animal that is tolerant of a homograft in one part of its body is tolerant in another; tolerance is systemic, and a tolerated graft does not build up a privileged position within its own lymphatic territory (§4·3).

Every degree of tolerance is possible, from that which allows a homograft to live only a few days beyond its normal median expectation of survival to that in which it is permanently accepted by and incorporated into its host. An inhibition of response which is partial may nevertheless be permanent, for the weakening of the 'secondary response' in partially tolerant animals is proportional to the weakening of the first (§§3·2, 4·4).

The stimulus which confers tolerance must be fully antigenic, i.e. must be one which would have caused an older animal to have become immune. Cells such as erythrocytes which have no power to elicit transplantation immunity are incapable of causing tolerance of tissue homografts; all treatments which abolish the power of cells to confer tolerance upon embryos will also abolish their power to make older animals immune (§5).

Immunological reactivity can be promptly and permanently restored to a tolerant animal by inoculating it with cells taken from the regional lymph nodes of actively immunized members of its inbred strain. It may also be restored, more slowly, by the inoculation of normal unimmunized lymph node cells. A tolerant mouse thus retains in full the power to give effect to an immunity of adoptive ('passive') origin; a tolerated homograft continues to be a source of antigenic stimuli, and its susceptibility to a reaction directed against it remains unchanged. Tolerance represents a central failure of the mechanism of the immunological reaction, and is not caused by an intercession at a peripheral level (§6).

The relationship between twinning, fertility, tolerance and red-cell chimerism is analyzed. Like dizygotic twin cattle, twin chicks that arise from double-yolked eggs are synchorial, are red-cell chimeras, and are tolerant of grafts of each other's skin. Tolerance and infertility are not causally connected. The tolerance produced in chicks by artificial synchorial parabiosis from the 10th day of embryonic life until hatching is accompanied by a true persistent red-cell chimerism. The disappearance of chimerism in partially tolerant chickens does not reveal a return to normal reactivity, for their secondary response to red cells reintroduced by cross-injection is profoundly impaired (§7).

Some measure of tolerance of skin heterografts may be achieved by the synchorial union of embryonic ducks and chicks (§8).

Tolerance may be produced by, and in respect of, tumour homografts, and by tumour homografts in respect of skin. A degree of immunity which does not suffice to hold in check the growth of a tumour may destroy a normal homograft completely; the growth of a tumour homograft is therefore a less exacting measure of tolerance than the survival time of a homograft of skin (§9).

A naturally acquired tolerance of maternal homografts is believed to occur, very rarely, in guinea-pigs, presumably by the accidental incorporation into a foetus of maternal cells. No such natural tolerance has been observed in mice or rabbits (§10).

Phenomena cognate with tolerance are considered. The partial inhibition of transplantation immunity which is caused by injecting adult animals with variously modified antigenic matter differs fundamentally from tolerance in mode of origin, for the substances which enhance the growth of homografts after administration to older animals have no power to confer tolerance upon embryos, and the substances which cause embryos to become tolerant merely cause adults to become immune (§11·1).

It is argued that the iso-antigens responsible for transplantation immunity should be sharply distinguished from those specialized end-products of differentiation which are iso-antigenic because they are potentially auto-antigenic, and which are potentially auto-antigenic because the antibody-forming system has no opportunity in normal development to become tolerant of their action (§11·2).

The phenomenon of tolerance is considered for its bearing upon the relationship between mammalian mother and foetus; upon the different stages of development at which immunity to different antigens may arise; upon the antigenic and genetic composition of the different tissues of a single individual; and upon the fate of iso-antigens in normal life (§11·3).

1. Introduction

The term 'acquired tolerance' will be used in this paper to describe a condition which—to outward appearance at least—may be regarded as the exact opposite of a state of acquired immunity. If a normal adult mammal or bird is exposed by grafting or inoculation to living cells derived from some other member of its own species, then (*a*) the foreign cells are destroyed, and (*b*) their recipient enters a state of heightened reactivity, as a consequence of which foreign cells transplanted to it on a later occasion are destroyed more rapidly than they were before. If, on the other hand, a mammal or bird is exposed to the influence of such a graft or inoculum during foetal life, then (*a*) the foreign cells survive, and (*b*) their recipient enters a state of lowered reactivity, by virtue of which cells of the same origin transplanted to it in adult life outlive their normal expectation of survival, and may never be destroyed at all. So it may come about that two adult mice of the same age, sex, upbringing and genetic origin, exposed beforehand to foreign cells of the same kind and in the same amounts, may react to the transplantation of a skin homograft in very different ways. In the mouse which received its prior inoculation when already adult, the skin homograft is reacted upon with special rapidity and vigour; in the mouse which was injected during foetal life, the homograft is reacted upon slowly and feebly, if at all. Both modes of response represent departures from normality, but the departures are in opposite directions, and the states of immunological competence which they reveal are of exactly opposite kinds. Nevertheless, they will be seen to have two important properties in common. Both are brought about by the exposure of animals to 'antigenic' stimuli, before or after birth as the case may be; and both are immunologically specific. The graft used for the test of altered reactivity must have an antigenic constitution identical with, or at least very closely similar to (§4·2), that of the inoculum which was originally responsible for the inception of the altered state.

The term *tolerance* will be used at present only in the sense of the foregoing description, i.e. to refer to a specific weakening or suppression of reactivity caused by the exposure of animals to antigenic stimuli before the maturation of the faculty of immunological response.

2. Previous work

The phenomenon of tolerance could be said to have been demonstrated by implication in a variety of experiments on embryonic birds carried out with quite different purposes in mind. The work of Eastlick, Willier, Rawles, Weiss and others (see, for example, Eastlick 1941; Rawles 1944, 1945, 1952; Weiss & Andres 1952) has shown that organ rudiments, skin, neural crest tissue, or pigmentary cells transplanted into embryos of the same or of different

species may survive beyond hatching, and even—when donors and recipients have been of the same species—into adult life. Danforth & Foster (1929) found that a certain not easily ascertainable percentage of skin homografts exchanged between newly hatched chicks of different breeds may survive for a year or more; but, as the age of the chicks increases, so the percentage of these abnormally long-lived homografts declines, to reach zero by the end of the second week (Cannon & Longmire 1952). As demonstrations of tolerance—which they were in no sense intended to be—all these experiments are open to the objection that the survival of the homografts might have been due to an antigenic adaptation of the grafted cells. Such, indeed, is the interpretation which Cannon, Weber & Longmire (1954) and Weber, Cannon & Longmire (1954) are inclined to read into their more recent experiments on skin transplantation in young and adult chickens. This interpretation, we shall later argue (§4·3), cannot be sustained. On the contrary, the work of Cannon and Longmire provides the best evidence so far available of the way in which tolerance gives way to the mature response, immunity, as development proceeds.

For the first clear demonstration of an example of tolerance we are indebted to the work of Owen (1945; see also Owen, Davis & Morgan 1946; Stone, Stormont & Irwin 1952). Twin cattle have long been known to be synchorial. By accurate serological discrimination, Owen was able to show that the great majority of twin cattle at birth are red-cell mosaics or chimeras, for each calf contains a mixture of its own red cells with red cells belonging to the cellular heritage of its twin. Evidently the twins exchange blood in foetal life through the anastomoses of their placental vessels. The state of chimerism lasts far beyond the life span of a red cell, and may be permanent; it follows, then, that red-cell precursors as well as red cells must have been exchanged in the foetal cross-transfusion and must have established themselves in the tissues of the opposite twin. The exchanged cells could hardly have undergone an antigenic adaptation, for they were in fact identified by their antigenic properties—the very properties which, according to a hypothesis of adaptation, should of necessity have been transformed. Outside cattle, red-cell chimerism seems to be a rarity; it has been described in one human twin, in whom it had persisted for 25 years (Dunsford, Bowley, Hutchison, Thompson, Sanger & Race 1953) and in one pair of twin lambs (Stormont, Weir & Lane 1953); but our own observations (§7·2) show that it may be the general rule in the twin chickens that arise from double-yolked and doubly fertile eggs.

It was next found (Anderson, Billingham, Lampkin & Medawar 1951; Billingham, Lampkin, Medawar & Williams 1952) that the majority of twin calves would accept homografts of each other's skin, without regard to differences of conformation, sex or colour; whereas grafts exchanged between siblings of independent birth (and in other close familial combinations) were very rapidly destroyed. The state of tolerance was shown to be specific, for a 'tolerant' calf accepted homografts from no donor other than its twin; and to extend to cells histologically far removed from those responsible for the inception of the tolerant state—to cells which, moreover, could have had no opportunity to adapt themselves to an alien soil. The tolerance was long enduring, and some of the subjects of these earlier experiments are still demonstrably 'graft hybrids' to-day.

The authors of this work expressed their intention of reproducing by experimental means, and in laboratory animals, the phenomenon which occurs by a natural accident

in twin cattle. The enterprise succeeded, as Burnet & Fenner (1949) had predicted, and its earlier results have been reported upon briefly by Billingham, Brent & Medawar (1953, 1955). A long-lasting state of tolerance was shown to be produced in mice, rats (see also Woodruff & Simpson 1955), rabbits and birds by the inoculation of their embryos with foreign homologous cells, and new evidence was brought forward of its systemic character and immunological specificity. Tolerance, it was found, could be summarily abolished by adoptive ('passive') immunization—clear evidence that it was due to a central failure of the mechanism of reaction, and not to some intercession at a peripheral level which merely denied immunity its power to take effect.

In the meantime other demonstrations of tolerance had succeeded elsewhere. Ripley (1953), working under the supervision of Dr R. D. Owen, established a persistent red-cell chimerism by the intravenous injection of foetal rats with haematopoietic cells, and Hašek (1953*a*, *b*) devised an ingenious technique for the artificial synchorial twinning of embryonic birds. Hašek found that chickens of different breeds which had been in vascular union across a chorio-allantoic bridge from about the tenth day of foetal life until hatching were incapable of responding to transfusions of each other's blood by forming iso-agglutinins, and later research (Hašek 1954) showed that the parabiotic partners would accept homografts of each other's skin. Hašek & Hraba (1955*a*, *b*) sought for, but were unable to find, evidence that parabiosis produced anything more than a transient red-cell chimerism; our own evidence (§7) shows that red-cell chimerism is a normal consequence of both natural and artificial synchorial twinning in embryonic birds, but that a weakening of the power to form iso-agglutinins in response to cross-transfusions may persist after a state of chimerism has disappeared. (The theoretical interpretation which Hašek has read into his work need not detain us; it purports, for example, to refute the 'absurd theory of genes'...'the unscientific and idealistic theory of genes'.)

This review has so far been confined to grafts of normal tissues and to iso-antigens. Tolerance also extends to malignant cells (Gross 1950*a*, *b*; Koprowski 1955; Bollag 1955; §9) and to grafts between members of different species (Simonsen 1955; Bollag 1955; §8). Gross found that although adult *C3H* or *C57* mice were entirely resistant to grafts of a leukaemic tumour indigenous to strain *Ak*, the tumour would usually grow after implantation into newborns—a valid example of tolerance produced by, and in respect of, malignant cells. Koprowski showed that an ascites tumour indigenous to mice of strain *C3H* would grow in mice of a normally resistant strain (*ICR*) which had been injected in foetal life with *C3H* blood. Tumours so transplanted underwent an antigenic transformation, as a result of which they could then be propagated in normal adult members of strain *ICR*. The transformation was due, in all probability, to a selection of the more compatible variants among the ascites population and not to an antigenic adaptation of individual cells (§4·3). Bollag inoculated the foetuses of rats with the tissues of mice, and found that their resistance in later life to the growth of a murine sarcoma was much impaired. Simonsen found that turkeys which had been injected *in ovo* with chicken blood had a much reduced power to respond to the injection of chicken erythrocytes by forming antibodies, and so conversely; and we ourselves find (§8) that grafts transplanted from chicks to hatchling ducks with which they had been in synchorial union may survive for longer than a month instead of for less than a week. It is potentially of some importance

that Simonsen, in collaboration with Dr R. J. C. Harris, found that turkeys which had been injected in embryonic life with the blood of chickens became susceptible to infection by the Rous fowl sarcoma agent—evidence compatible with the belief that the Rous virus contains a 'chicken-specific' antigenic component.

Tolerance may well be a phenomenon of general immunological significance. Burnet & Fenner (1949) pay special attention to the work of Traub (1936, 1938, 1939), who found that mice exposed *in utero* to the virus of lymphocytic choriomeningitis were incapable, in later life, of forming neutralizing or complement-fixing antibodies.* (As it happens, the mice become passive carriers of the infection, transmit it to their offspring in foetal life, and show no signs of illness; they are therefore 'tolerant' of the virus in a different, though more familiar sense.) Buxton (1954) found that the intravenous injection of killed *Salmonella pullorum* organisms into chick embryos not more than 15 days old reduced their power to resist a later, oral challenge. Calves up to 4 weeks old produce no antibody in response to the intramuscular injection of *Trichomonas foetus* antigen, and, if the doses are large, their power to do so in later life is seriously impaired (Kerr & Robertson 1954). Rabbits exposed in very early life to moderate (i.e. to ordinarily effective and not overwhelmingly large) doses of bovine serum albumin not only failed to produce precipitating antibodies, but were still unable to do so when 4 months old (Hanan & Oyama 1954). A similar principle is revealed by the work of Dixon & Maurer (1955). More recently, Cinader & Joubert (1955) found that rabbits injected from birth onwards with human albumin remained entirely unresponsive to active immunization by human albumin for at least 8 months; and when rabbits which had been injected with human albumin at birth were later challenged with a modified antigen, viz. human albumin coupled with the diazo compound of benzene-*p*-sulphonic acid, the recipients responded (when they responded at all) by forming antibodies directed specifically against the inserted determinant group. The specificity of acquired tolerance induced by 'non-living' antigens can therefore hardly be in doubt.

So much stands to the credit of the idea that tolerance may be a general property of immunological reflex systems. On the other hand, Burnet, Stone & Edney (1950)—in experiments designed to reveal the phenomenon of tolerance, if any such phenomenon should exist—found that the injection of chicken embryos with living influenzal or bacterial virus, or with human red cells, did not abolish or weaken the reaction against these antigens after their administration for a second time when the chicks were 6 to 9 weeks old. In the light of more recent knowledge, these negative findings cannot be given their apparent weight. The routes of injection of the viruses (into the yolk-sac or allantois) may well have been unsuitable, for Buxton found only the intravenous route to be effective; the human red cells were injected intravenously, but once only, and in a comparatively low dosage; and with the destruction or dying off of the red cells the antigenic stimulus would presumably have come to an end. In the experiments of Hanan & Oyama, the antigenic stimulus was administered repeatedly, and in a manner designed for slow absorption and therefore for prolonged effect.

There are therefore reasonable grounds for believing that the phenomenon of tolerance is by no means confined to iso-antigenic systems or to immunity against living somatic cells.

* See also Komrower, Williams & Stones (1955).

Nevertheless, experiments on the induction of tolerance that make use of the 'homograft reaction' have distinctive technical merits. The antigenic stimulus to which the embryos are subjected is powerful, innocuous and persistent, and it arises from cells which may be accepted as functional and anatomical parts of the tissues of their host. The homograft reaction itself is perhaps the most accurately reproducible of any studied in immunology, and the measurement of its intensity, expressed in terms of the median survival time of homografts, has the degree of precision represented by a standard deviation of only about $\pm 10\%$ (Billingham, Brent, Medawar & Sparrow 1954).

3. The induction of tolerance

3·1. *Outline of principles*

Tolerance of homoplastic grafts may be brought about by the exposure of embryos or very young animals to what, in adults, would be an effectively immunizing dose of foreign homologous cells. The donor of the foetal 'graft' or inoculum may be an adult, or may itself be an embryo, but in either event the inoculum prepared from it must consist of living, or at least of antigenically active, cells (§5). The age of the injected embryo may range between a lower limit set by technical obstacles and an upper limit which must be empirically found.

The injected embryos are allowed to hatch or to be born. When they have reached a size which is technically convenient for grafting, and which is known from normal animals to be one at which they would be immunologically mature, they are grafted for a second time. The second graft (*test graft*) must come either from the donor of the original inoculum or from an animal containing no antigens not also present in the original donor—for example, from some other member of the donor's inbred strain.*

The test graft has one of three possible fates before it: it may (*a*) fall short of, (*b*) come up to, or (*c*) exceed its normal expectation of survival, which will be known beforehand. If the earlier grafting or inoculation has conferred immunity, the first of these possibilities will be realized, and if tolerance, the third. The second possibility corresponds *ex hypothesi* to a state of affairs in which the net effect of the prior treatment has been zero.

In our experiments, the tissue chosen to provide the test homograft has almost invariably been skin. The use of a skin homograft makes for a test of the greatest sensitivity, and one of which the results are open to outward inspection from day to day. The behaviour of a rapidly growing tumour homograft can give a very misleading impression of the degree of tolerance and of the efficacy of the methods used to produce it, for quite a short prolongation of its normal lifetime will allow a transplanted tumour to build up a momentum of growth which is quite beyond the power of the homograft reaction to oppose (§§9, 11·1). A skin graft, by contrast, will show up very clearly a reaction that is too feeble to destroy the grafted cells. It does so by imperfect differentiation, partial baldness, a tendency to build up fibrous tissue and to undergo contracture, and an eczematous deterioration of the epithelial surface. Such behaviour is characteristic of skin homografts on mice which are not quite fully tolerant. It corresponds exactly with the behaviour of skin homografts exchanged between animals that are almost but not quite antigenically identical—

* The relationship is discussed more fully in §4·2.

e.g. between mice belonging to separate sublines, of recent common origin, of a single highly inbred strain (Billingham, Brent, Medawar & Sparrow 1954). In short, the survival time and the cosmetic properties of a skin graft can be relied upon to show up the whole spectrum of compatibility, not excluding the feeblest immunity or (which comes to the same thing) a tolerance which is almost but not quite complete.

3·2. *Induction of tolerance in foetal mice*

The peculiar advantage of mice for studies on the phenomenon of tolerance is that they exist in a wide variety of highly inbred strains. The experiments described in this section were intended to provide the tolerant mice needed in all the analyses (§§4, 6) for which inbred animals were indispensable. For investigations of other kinds—e.g. on the properties of the inoculum conferring tolerance—chickens are much to be preferred (§5). The present section is confined to the induction of tolerance in foetal mice by, and in respect of, normal tissues. Newborn mice (§4·1) and cancerous tissues (§9) are dealt with later.

Subjects; control data

The mice we have used belong to domestic sublines of three inbred strains: *A*, *AU* and *CBA*. Strain *A* is white (albino), strain *AU* black, and strain *CBA* brown (agouti). The median survival times of skin homografts transplanted between normal adult members of these sublines according to our standard surgical procedure are summarized in table 1, taken from the data of Billingham, Brent, Medawar & Sparrow (1954).

TABLE 1. MEDIAN SURVIVAL TIMES OF HOMOGRAFTS EXCHANGED BETWEEN ADULT MEMBERS OF DIFFERENT INBRED STRAINS OF MICE

strain combination	MST (days)	standard deviation	standard error
$A \rightarrow CBA$	11·0	1·1	0·3
$CBA \rightarrow A$	10·2	0·9	0·3
$A \rightarrow AU$	9·0	0·9	0·3
$AU \rightarrow A$	9·1	1·4	0·4

The median survival time (MST) may be regarded as invariant for differences of age within the range 6 weeks to 1 year and for difference of sex. It is, however, profoundly influenced by a pre-existing state of immunity or sensitivity. In all strain combinations, a second homograft transplanted 15 days after a first is destroyed within 6 days of grafting. As the time interval between the first and second transplantations increases, so the state of immunity slowly decays; but in the strain combination $A \rightarrow CBA$ (that most fully studied), the MST is still only about 6 days after an interval of 240 days between the first and second graftings (Billingham, Brent & Medawar 1954, and unpublished). For all practical purposes this means that an adult mouse, once immunized, does not return to a state of virgin reactivity in its lifetime.

The appearance of a 'control' skin homograft, 11 days after transplantation to a normal mouse, is illustrated by figure 2, plate 7.

Preparation of inocula

The inocula which we have used for the induction of tolerance include adult whole blood and concentrates of leucocytes prepared from it; tumour grafts (§9); and cellular suspen-

sions prepared from a variety of tissues, but most usually from a mixture of kidney, liver, testis and spleen. In mice (unlike birds, §§3·3, 5, 8) whole blood and its derivatives gave decidedly inferior results, for reasons which will be mentioned later; and we found no evidence that embryonic tissue-cell inocula were superior to those prepared from adults.

Blood was withdrawn directly from the heart into a syringe containing not more than 0·1 vol. of 4% trisodium citrate. Leucocyte concentrates were at first prepared by resuspending the buffy coat of spun blood in an appropriately reduced volume of citrated plasma; but latterly by the technique of Minor & Burnett (1948), which depends upon the removal of red cells by accelerated sedimentation. For this purpose, cardiac blood was received into a syringe containing not more than 0·1 vol. of a 10% solution in distilled water of bovine plasma Fraction I (Armour), a dry powder containing about 40% fibrinogen and 40% sodium citrate by weight. The red cells were allowed to settle naturally for 60 to 120 min, and the supernatant fluid, free from red cells, was then withdrawn.

Tissue suspensions were prepared either by a prolonged chopping of the chosen organs with sharp round-ended scissors under normal saline or Ringer's solution, or by pressing the coarsely divided organs with a flat-bottomed glass pestle through a fine-meshed stainless steel sieve (250 to 300 holes/cm^2). Much of the cellular debris was cleared from the diluted preparation with a single gentle centrifugation; the sediment (consisting of isolated cells and small organized fragments of tissue) was taken up in a smaller volume of fluid to form a dense suspension, and then drawn into a hypodermic syringe through the needle later to be used for inoculation. It seemed to us preferable to use a fine needle, of internal bore not exceeding 0·25 mm, although, with the older embryos, a wider needle (0·4 mm) has been used without apparent ill effect.

The final cellular concentration was of the order of 500000 cells/mm^3; an accurate assessment was hardly practicable, for not all the cells were separate.

The dosages administered to embryos of different ages were controlled by a micrometer attachment, and were usually as follows: 15-day embryos, 5 mm^3; 16- to 17-day embryos, 10 mm^3; 18-day and older embryos, 20 mm^3. (For adult animals, these are strongly immunizing doses: see §5·1.) Volumes larger than 30 mm^3 were not well tolerated, even by the older embryos.

Injection of embryos

Mice are best injected on the 16th or 17th day of foetal life, for this represents the most satisfactory compromise between what is immunologically desirable (the earlier the better: see §4·1) and what is surgically expedient (the reverse). Embryos injected as early as the 15th day of foetal life almost invariably undergo abortion. The rate of recovery of living newborns injected 24h or even 12h before birth is higher than with mice injected at 16 to 17 days, but the proportion which enjoy a *high* degree of tolerance is somewhat lower; it may be inferred that at this later age a higher proportion have entered the 'null period' (§4·1) which precedes the onset of the adult mode of response. Mice at the appropriate stage of pregnancy are most economically provided by time-controlled matings or by inspection for well-formed vaginal plugs. (The palpation of embryos or the mere sizing-up of a pregnant mouse can be misleading guides to the stage of pregnancy.) Multiparous

mice make better mothers than mice in their first pregnancies. Mice intended for operation are segregated 3 or 4 days beforehand, given plenty of nesting material, and maintained on a diet of mixed grain and rat cake, supplemented by diluted milk.

The surgical procedure is quick and simple (figure 1, plate 7). Each mouse is anaesthetized with pentobarbitol sodium ('Nembutal') in a dosage of 70 mg/kg intraperitoneally, and tied out on its back by means of elastic bands attached to its four legs. A median ventral strip of fur is removed with fine mechanical clippers, taking care not to touch the nipples. The skin is prepared with a mild antiseptic in a fatty base, mainly to fix the hairs, and then opened with a $3\frac{1}{2}$ to $4\frac{1}{2}$ cm incision in the ventral midline. The edges of the skin are neither mobilized nor undercut; they gape sufficiently when the fascial planes overlying the body wall have all been severed. The body wall itself must not be cut, but it should be moistened from time to time with normal saline.

In systematic order, as many embryos as possible are brought into view by gentle finger-tip manipulation; not all are necessarily accessible to injection, and one or two may be accidentally overlooked. Indeed, a comparison between the number of foetuses injected and the average litter size in our normal breeding colonies showed that as many as a quarter of the embryos present may escape injection—a figure to which we should have been able to give greater precision if we had adopted Koprowski's (1955) method of colouring the inoculum with a vital dye such as trypan blue.

Each foetus is injected intra-embryonically in the trunk region, the entire inoculum being administered with a single forwardly directed insertion of the needle. The intra-amniotic injections resulting from too shallow an insertion of the needle, being ineffective, should be avoided; but an equally serious error, often difficult to avoid, is to insert the needle too far. After injection the skin edges are reunited with closely spaced interrupted sutures; the mouse is allowed to recover from the anaesthetic in an incubator, and then returned to its nest. As with all pregnant mice, the less they are interfered with thereafter the better, whether before or in the few days immediately after birth.

With inbred mice, such as we were obliged to use, the number of viable newborn offspring born of injected mothers was often disappointing; some embryos underwent abortion or were born prematurely, others were born alive but maltreated by their mothers, and others still were inadequately reared. Twelve complete litters were lost from the last twenty-five pregnant mice we operated upon. On the other hand, the maternal mortality, being well below 1 % of all subjects, is negligible.

The question of what happens to the foetal inoculum in mice has not been answered. 'Rests' of inoculated tissue have only rarely been found. On the other hand, there is no reason to doubt that at least some cells of the foetal inoculum survived for as long as the tolerant state which they were responsible for creating; and this supposition is borne out by the persistence of red-cell chimerism in parabiotic chickens (§7) and dizygotic cattle twins (see §2).

Results: (a) with tissue inocula

Six to eight weeks after its birth, each injected mouse was challenged with a skin homograft from a member of the same strain as the donor of the original inoculum. The many advantages of an earlier test (e.g. at weaning) were sacrificed to surgical convenience.

In no mouse did the test homograft fall significantly short of its median expectation of survival; no subject could therefore be held to have been sensitive or immune. Regardless of the strain combination of donor and recipient, the grafts were scored as showing the lowest measurable degree of tolerance when they were still alive 14 days after grafting, i.e. at about three standard deviations' distance above the normal value—a figure which, in many hundreds of grafting operations in our three standard strains, has never yet been reached by any inter-strain homograft on a normal adult mouse.

We may first consider the results of injecting suspensions of cells prepared from normal organized tissues (see figures 3 to 5, plate 7), disregarding mice injected with leucocytes or whole blood; and these experiments may again be subdivided into those in which the injections were carried out at the 18th day of foetal life or later, and those in which the injections were done earlier than the 18th day.

TABLE 2. INDUCTION OF TOLERANCE IN FOETAL AND NEWBORN MICE BY INJECTING ADULT TISSUES AND ADULT WHOLE BLOOD (§§3·2, 4·1)

inoculum	age of recipients	no. tested	no. tolerant	tolerated grafts surviving > 50 days
adult tissue (all expts)	foetal, < 18 days	93	40 (43 %)	23 (58 %)
	foetal, ⩾ 18 days	49	23 (47 %)	3 (13 %)
	all foetuses	*142*	*63 (44 %)*	*26 (41 %)*
	newborn mice	94	8 (8½ %)	5 (63 %)
adult tissue (last 25 expts)	foetal, < 18 days	29	17 (59 %)	14 (83 %)
	foetal, ⩾ 18 days	12	6 (50 %)	2 (33 %)
	all foetuses	*41*	*23 (56 %)*	*16 (70 %)*
adult blood (all expts)	foetal, < 18 days	69	11 (16 %)	1 (9 %)
	foetal, ⩾ 18 days	36	2 (6 %)	0
	all foetuses	*105*	*13 (12 %)*	*1 (1 %)*

The number of mice in the later foetal age group showing any degree of tolerance, expressed as a proportion of the number which survived to be tested, was 23/49 or 47 % (see table 2). The corresponding figure for the earlier age group was 40/93 or 43 %. The difference, as it stands, is insignificant, but it cannot be accepted at its face value. The older embryos are much the easier to inject with certainty, and in the older age group a higher ratio of injected to non-injected mice may be assumed to have survived. Moreover, the proportions of tolerant mice in the older and younger age groups showing a *high* degree of tolerance—the survival of the test homograft in an entirely normal condition for upwards of 50 days—were 3/23 = 13 % and 23/40 = 58 % respectively, a highly significant difference. It follows that the earlier foetal injections probably give a higher proportion of tolerant mice, and certainly a higher degree of tolerance, than the later.

That the proportion of mice showing any degree of tolerance is so low (63/142 = 44 %) may be attributed to a combination of the following sources of error: (*a*) about one-quarter were probably not injected *in utero* at all; among those which survived to be tested, the proportion of uninjected mice may have been still higher, because of a differential mortality weighted in their favour. To this may be added (*b*) the occurrence of frankly inept injections which were not, as they were intended to be, intra-embryonic, and (*c*) the fact that among the injected embryos there must have been a number in which the tissue inocula were deposited in inhospitable or immunologically unsuitable positions of the embryo. The second and third of these defects were to some extent remedied. In the last

twenty-five pregnant mice we operated upon (see table 2) the percentage of tolerant mice showing a high degree of tolerance rose to 83 % in the younger foetal age group and 33 % in the older, but the proportion of tested mice which showed any degree of tolerance remained very much as before.

Results: (b) inoculation of blood

The results of using adult whole blood or blood leucocytes for foetal inoculation were much inferior, both as to the number of tolerant mice recovered and as to the degree of tolerance achieved, as consultation of table 2 will show. Our best example is illustrated by figure 6, plate 7. The effectiveness of whole blood when injected into foetal (§3·3) and even newborn (§5) chicks, and in parabiosis (§7) of chick embryos, may therefore be attributed to its injection by the intravenous route. Dosage must also be a factor. Compared with tissue inocula, the effective dosage of blood to which foetal mice can be exposed is very meagre; the active ingredient of blood is in the leucocyte fraction (§5), so that a normal inoculum of 10 mm^3 can contain a maximum of only about 50000 active cells. Embryonic chickens received doses up to twenty-five times larger.

Duration of tolerance; 'life table'

The tolerance produced by living cells may be virtually permanent, possibly because they provide a continuing antigenic stimulus which ensures that the state of tolerance is self-maintained. Dead cells elicit neither tolerance nor tissue transplantation immunity (§5); the duration of the tolerance produced by a *transient* exposure of embryos to an iso-antigenic stimulus is therefore not yet known. But of its permanence when living cells are used there can be no question. 'Mrs McK', the human red-cell chimera described by Dunsford *et al.* (1953) is now 28 years old; Rawles (1944, 1945) has described inter-breed chimeras, in birds, of 3 or 4 years' standing—chimeras produced by methods which, in our interpretation, rely upon the principle of tolerance; and the natural tolerance of each other's skin grafts in dizygotic twin cattle is known from the unpublished work of Lampkin to last longer still. Moreover, even when tolerance is incomplete, so that a test homograft is ultimately rejected, the resistance of the host to later homografts remains impaired (§4·4). In mice we can show very few examples of really long-standing tolerance, because the great majority were used for experimental analysis when the test grafts were upwards of 50 days old, but we have at present four mice with homografts of more than 200 days standing, on two of which the grafts are 450 days old.

The longer a homograft is tolerated, the more likely it is to survive for some further, arbitrarily chosen, period of days; the mean expectation of life progressively increases as the graft grows older. Grafts are held to reveal tolerance (see above) when they have survived for 14 days; the numbers which underwent regression within three successive intervals of 50 days thereafter, expressed as a percentage of those still alive at the beginning of each interval, were 60, 26 and 11 % respectively—figures based upon mice injected *in utero* with inocula prepared from tissues, as opposed to blood. The last two figures represent gross overestimates of the 'force of mortality', for the best grafts—those in which there was no hint of impending deterioration—were always selected for experimental treatment (§§4, 6) and could not therefore take their place in the life table.

The regression of a long-tolerated graft is not an acute phenomenon, but a long-drawn-out deterioration which may well occupy 20 to 30 days. It begins with a pinkness symptomatic of a chronic state of mild vascular dilatation and congestion. Small superficial scabs or other blemishes appear on the epithelial surface, patches of hair are lost, and the graft as a whole contracts. Normally the process ends with the scabbing of the entire epithelial surface, but in borderline cases (as we have already mentioned) a mildly eczematous state may persist indefinitely, and may even from time to time improve. A graft is not held to give evidence of *complete* tolerance unless it is supple, fully haired, and uncontracted; has an epithelial surface with a normal 'grain' and texture, and a normal pattern of dermal connective tissue fibres—in short unless it is 'autograft-like' in every way. Needless to say, a tolerated graft retains the outward evidence of its genetic origin in the distinctive pattern and coloration of its hair.

3·3. *Induction of tolerance in embryonic birds*

Birds are well suited in several ways to experiments on the induction of tolerance, because to all their other embryological advantages they add the accessibility of the intravenous route of inoculation. Moreover, their immunological responses are as lively and versatile as those of mammals, and they appear to be no less rich in iso-antigenic variants. Birds have accordingly been used to investigate the properties of the stimulus which confers tolerance (§5), the relationship between tolerance and red-cell chimerism (§7), heterologous tolerance (§8), and a number of other problems besides (§4). The present section is confined to the induction of tolerance in embryos by the intravenous injection of adult or foetal blood or by the chorio-allantoic grafting of adult tissues.

Subjects; control data

The donors and recipients of our experiments were Rhode Island Red (*RIR*) and White Leghorn (*WL*) chickens respectively. All test operations were carried out when the recipients were 14 days old from hatching (figures 8, 9, plate 7). Twenty-nine out of thirty skin homografts transplanted from 2-week-old *RIR* donors to 2-week-old *WL* recipients were found to have been totally destroyed within 9 days of their transplantation (figure 10, plate 7), the one exceptional graft surviving to only the 16th day. These results are entirely in keeping with those of Cannon & Longmire (1952), and they show that, in its power to reject a graft of foreign homologous tissue, a 2-week-old chicken may be regarded as immunologically mature. It is, however, important (see §§4·3) to notice that grafts from *adult* donors on 2-week-old recipients enjoy a slightly longer expectation of survival; these results, discussed in §4·3, are set out in §5.

In spite of their outward uniformity in respect of breed characters, the *RIR* donors were a highly heterogeneous assembly; twenty-three out of thirty-one 'within-breed' homografts transplanted between 2-week-old *RIR* chicks were destroyed within 10 days of transplantation, and only three survived beyond the 20th day. The donor of the foetal inoculum in each experiment was therefore of necessity the donor of the later test graft; in chicks, by contrast to inbred mice, no donor could deputize for another. The success of an experiment in which the blood of one embryo was transfused into another depended, therefore, upon their *both* remaining alive until the day of testing. With the adult donors

of tissues used for chorio-allantoic grafting, this was clearly impossible; grafts were therefore removed from one donor on the day of the foetal inoculation and, after impregnation for 1 to 2h at room temperature in a 15% v/v solution of glycerol in Ringer's solution, were slowly frozen to $-79°$ C and kept at that temperature awaiting use (Billingham & Medawar 1952).

Intravenous inoculation

All the recipients were 10- to 11-day-old *WL* embryos, prepared for intravenous inoculation by the method described by Beveridge & Burnet (1946). The exact position of, and the direction of blood flow within a prominent chorio-allantoic vein was marked on the surface of the shell by candling, and a suitably placed 8×12 mm window was cut in the shell with a mechanical drill. A drop or two of paraffin oil on the exposed shell membrane brought the vessel into prominence, whereupon it was entered with a fine hypodermic needle inserted in the direction of blood flow and, as nearly as possible, coaxially with the vein (figure 7, plate 7). The volume injected ranged from 50 to 250 mm^3. Withdrawal of blood from a donor embryo was done by an exactly similar method except that the needle was inserted against the direction of flow. The clotting of blood was prevented by lubricating the syringe beforehand with liquid paraffin. Blood was withdrawn from adult donors through the wing vein, without the use of anticoagulants; it remained stable in a chilled siliconed vessel for the hour or two before it was used. After injection, the hole in the shell was sometimes sealed with a rectangle of sticky cellulose film tape, but at other times, without apparent disadvantage, left uncovered.

Having reached the 14th day from hatching, seventeen *WL* chicks were grafted with skin from their respective *RIR* donors by the method of Cannon & Longmire (1952) (figures 8, 9, plate 7). Fourteen of the homografts lived at least twice as long as the normal expectation of survival; seven of the fourteen were still surviving at the 60th day, and three of the seven at the 120th day, when shortage of cage space obliged us to dispose of them. Six of these seven well-tolerated grafts grew a normal crop of donor-specific feathers (figure 11, plate 7).

The experiments in which *adult* blood was injected into embryos met with about 95% mortality, and only the odd survivors allowed us to establish the principle that injection of adult blood can confer long-lasting tolerance, revealed by homograft survival times of more than 120 days. Deaths occurred towards the end of incubation, i.e. 4 to 5 days after inoculation; the fact that the blood of adult ducks was usually inoffensive, and that not all chicken donors were guilty, suggests an infective cause of death. The mortality from injecting blood cells washed in normal saline was very much lower; but when it became clear that tolerance could be induced by injecting *newly hatched* chicks with adult whole blood (§5), our experiments on embryonic injection were abandoned.

Chorio-allantoic grafting

Eight 10- to 11-day-old *WL* embryos were grafted upon the chorio-allantoic membrane with small fragments of a miscellany of adult tissues (kidney, spleen, heart, lung) suspended in Ringer's solution. The inoculum was introduced through a small circular window in the shell overlying a richly vascular area of the chorio-allantoic membrane; it was found

helpful to 'drop' the membrane, according to the usual practice, by making a tiny hole in the air chamber and withdrawing a little air. Fourteen days after hatching the eight *WL* chicks were grafted with the donor's skin, preserved by freezing. Four of the eight grafts survived for more than 20 days after grafting, and of these three survived for upwards of 60 days. These experiments show that inoculation by the chorio-allantoic route is indeed capable of inducing tolerance.

3·4. *Induction of tolerance in foetal rabbits and rats*

Rabbits were not available to us in inbred strains of proven iso-antigenic uniformity, and at least one strain of rats inbred on the principle of the 'closed colony' is known to be antigenically diverse (Billingham & Parkes 1955). With both rabbits and rats, therefore, as with chickens—and for exactly the same reasons—the donor of the foetal inoculum had also to be the donor of the test graft. The experiments summarized in this section deal with the injection of foetal rats and rabbits with whole blood or leucocyte concentrates from adult donors.

Blood from rabbits was withdrawn through the median ear artery and from rats by cardiac puncture; it was treated by methods which have already been described (§3·2). The techniques of skin grafting in rabbits and rats were those of Billingham & Medawar (1951) and of Billingham & Parkes (1955) respectively. The donors, and the pregnant females whose foetuses were to be injected, were chosen for the greatest possible dissimilarity of origin, colour and conformation. Under these conditions, the median survival time of skin homografts on adult rats and rabbits is known to be below 10 days (see the evidence summarized in §7 of the first paper of this series).

Rats

The foetuses of rats and rabbits can be injected reliably only by exposing the uterus through a laparotomy incision; the viscera should be handled with particular care and not touched with metal instruments. The foetuses of ten females in the 16th to 21st day of pregnancy were injected intraperitoneally with 20 to 100 mm^3 whole blood; the embryos, as a rule, were easy to see. Three litters were lost by maternal neglect or death *in utero*. From the remaining seven, forty-three rats survived to the age of test operation—a yield which compared very favourably with that from inbred mice. As before, however, the results of using blood were fitful. The members of four litters (twenty youngsters) showed no tolerance at all; all the test homografts were destroyed within 12 days. The survival scores of the homografts on the twenty-three members of the three remaining litters are summarized in table 3*A*. Litter 3, the most successful, was the only one to have been injected *in utero* with a leucocyte concentrate. These results confirm the inferences drawn from similar experiments with mice (§3·2) that adult whole blood can confer tolerance, but very unreliably, presumably because it cannot be injected by the intravenous route. In a purely surgical sense, the rat is a good subject for experiments on tolerance.

Rabbits

Our results with rabbits were much the same as those with rats, but at the relatively earlier stage of gestation which we used (with one exception, 17 to 22 days) the embryos

were much more difficult to see and therefore to inject reliably. The foetuses of eleven pregnant females were injected with 20 to 100 mm^3 of adult blood. One litter was lost completely, and in each of three others only one rabbit survived. Disregarding these four litters, twenty-nine rabbits representing seven litters survived to the age of the test operation. Eleven in twenty-nine, or about one in three, showed some measure of tolerance, and all eleven belonged to three litters; the homografts on the fifteen members of the remaining four litters (three of which had been injected with leucocyte concentrates) were totally destroyed within less than 12 days. The survival scores of the members of the three successful litters are entered in table 3*B*.

Maternal mortality in both rats and rabbits was nil.

TABLE 3. SURVIVAL TIMES OF HOMOGRAFTS ON MEMBERS OF THREE LITTERS OF RATS (*A*) AND OF RABBITS (*B*) AFTER INJECTION OF ADULT WHOLE BLOOD OR OF CONCENTRATES OF BLOOD LEUCOCYTES

For description of other litters, see text (§3·4).

	litter	inoculum (mm^3)	foetal age (days)	survival times of homografts (days)
(*A*) rats	1	whole blood, 20	18	<12, <12, <12, <12, <12, <12, <12, *44*, *60*
	2	whole blood, 20	$19\frac{1}{2}$	<12, <12, <12, <12, $\gg$*11* (died), *23*, *26*, $>$*191*, $>$*191*
	3	leucocytes, 30	21	$\gg$17, $>$*140*, $>$*140*, *140*, $>$*140*
(*B*) rabbits	1	whole blood, 50	22	<12, <12, *180*
	2	whole blood, 50	21	<12, *14*, $>$*28*, $>$*28*, *39*, *42*
	3	leucocytes, 50	20	*13*, *14*, *22*, *36*, *80*

4. TIME RELATIONS; SPECIFICITY; ADAPTATION; THE SECONDARY RESPONSE

4·1. *Time relations; the 'null period'*

In §5 it will be shown that the stimulus which causes embryos or very young animals to become tolerant is one which would have caused older animals or adults to have become sensitive or immune. At some epoch of life, therefore, there must be a transition from the one modality of response to the other. The transition could hardly be a sudden one; it will be shown that the power of an 'antigenic' stimulus to confer tolerance falls away to zero as the age of the injected subject increases, and it is, of course, a commonplace of immunology that power to respond to an antigenic stimulus by immunity rises from zero to its adult value as development proceeds (see Burnet & Fenner 1949). In our immunological system, tolerance and immunity are revealed by a test graft's exceeding or falling short of its median expectation of survival, i.e. the median lifetime of homografts on normal adult hosts. A survival time not significantly different from its normal median value therefore represents the zero of both scales of measurement, so that, in a purely numerical sense, tolerance may be thought of as negative immunity or immunity as negative tolerance. Presumably, therefore, there must be some age or range of ages at which the presentation of an antigenic stimulus confers neither tolerance nor immunity—at which the net effect of exposure is nil.

Evidence has already been given (§3·2) that mouse embryos younger than 18 days are superior to older embryos in respect of the proportion which become tolerant and the degree of tolerance which is achieved. The reactions of newborn mice confirm this trend

(table 2). A total of ninety-four newborn mice from eighteen litters were injected 2 to 12h after birth with adult tissue preparations of the same kind as those used for the injection of embryos, except that the doses were proportionately larger (30 to 40 mm^3) and were usually administered by both intraperitoneal and subcutaneous routes. (Subgroups of mice which received additional treatments are mentioned later.) Only $8/94 = 8\frac{1}{2}\%$ were in any degree tolerant, and five of these belonged to a single litter, the treatment of which was in no way out of the ordinary. The survival times of the homografts on these five mice were 32, 64, 128, 168 and >170 days. On not one of the remaining eighty-six mice did the survival time of the test homograft fall significantly *short* of the normal value. So far as homografts are concerned the epoch of birth represents, for the great majority of mice, a *null period* during which exposure to an antigenic stimulus confers neither tolerance nor immunity to any appreciable degree. The inoculation of newborn mice with foreign homologous cells either confers tolerance or, more usually, has no perceptible effect at all. The newborn rat is a more favourable subject (Woodruff & Simpson 1955).

One subgroup of twelve newborn mice received ten or eleven successive inoculations, at 3-day intervals, of quantities increasing finally to 70 to 90 mm^3. None was immunized by this treatment; in one, the test homograft survived for more than 70 days. A second subgroup of fifteen mice received three subcutaneous injections of 0·05 mg cortisone acetate at 2-day intervals in addition to the tissue inoculum. Growth and the onset of pigmentation were severely retarded; one mouse alone was tolerant. Inasmuch as the administration of cortisone prolongs the life of homografts in adult animals (see §11·1), and does so by a central suppression of the immunological response, it was hoped that the administration of cortisone to embryos would delay the maturation of the antibody-forming system and so prolong the epoch during which they would respond to antigens in the embryonic fashion. Cannon & Longmire (1952) found that the injection of newly hatched chicks with cortisone increased the percentage of long-term survivors among skin homografts transplanted from other hatchlings. Our own failure to reveal any such phenomenon is probably due to the fact that chickens are immunologically less mature at birth than mice: contrast the meagre percentage of tolerant mice obtained in the present experiment with the results of injecting newborn chicks (§5).

Taken at their face value, the entries in table 2 suggest that exposure to an antigenic stimulus confers tolerance on about 45% of foetal mice and 10% of newborns. The estimates are, however, heavily biased in favour of the newborns. Newborn mice can be injected with certainty and in known positions. The ostensible value for foetal mice must therefore be raised to allow for imperfect injections and for mice—about one-quarter of the total number: §3·2—which were not injected at all.

The best evidence for the progressive decay, with increasing age, of the power of an antigenic stimulus to confer tolerance is that which can be read into the work of Cannon & Longmire (1952) on newly hatched chickens. Homografts were exchanged between pairs of chicks of different breeds and of steadily increasing ages. The proportion which tolerated their homografts for 2 months or more fell from 16% at hatching to zero 2 weeks later. From our point of view this work is open to the objection that the age of the donors, which should be a parameter of the experiment, increased in step with the hosts' age. In later

work, however, Cannon *et al.* (1954) fixed the age of the donors at 1 day, and found that 3/30 homografts on 1-day-old hosts survived 6 months or more, contrasted with 0/176 when the hosts were upwards of 7 days old. In our own experiments, we found that $14/17 = 82\%$ of 10- to 11-day-old *WL* embryos became tolerant in response to intravenous injections of whole blood (§3·3), as opposed to $20/46 = 43\frac{1}{2}\%$ of newborn chicks injected with whole blood or with blood leucocytes (§5, table 7). The experiments are, however, not quite comparable, because the foetal chicks received inocula of embryonic blood and the newborn chicks received adult's.

The experiments on newborn chicks to be described in §5 lead to one conclusion that is immediately relevant. The survival times of the homografts transplanted to injected chicks sometimes exceeded, but never fell below, the survival times of homografts on the uninjected controls. In other words, the injection of blood into newly hatched chicks *either* produced tolerance *or* produced no perceptible effect at all. Thus chicken hatchlings, like newborn mice, are in, or on the threshold of, the 'null period'; the majority have lost the power to respond to antigens by becoming tolerant, but have not yet acquired the power to react by becoming immune.

It may be concluded, then, that the response of animals to foreign homologous cells changes from tolerance to immunity in course of development, and passes through a 'null period' on the way—a short epoch of life in which the net effect of prior treatment is such that the adult subject behaves as if it had received no prior treatment at all.

4·2. *Specificity*

The specificity of acquired tolerance may be considered under two quite distinct headings: (*a*) 'tissue specificity', i.e. the power of one tissue to confer tolerance of another tissue from the same individual; and (*b*) 'individual specificity', the power of tissues from one individual donor to confer tolerance of tissues taken from another. Both forms of specificity turn ultimately upon differences of antigenic constitution, of developmental or genetic origin, as the case may be. In what follows, the word 'antigen' is used as shorthand for 'iso-antigen of a kind capable of eliciting transplantation immunity'; many of our conclusions are quite inapplicable to iso-antigens of other kinds (§11·2).

Tissue specificity

It is a well-established fact that, in transplantation immunity, one living tissue can provoke an immunity which is visited upon another. In rabbits, for example, an intradermal injection of leucocytes will heighten the resistance of their recipient to a later homograft of skin (Medawar 1946*b*); in rats, a skin homograft will make its host refractory to an endocrine ovarian graft (Billingham & Parkes 1955). Such experiments can only show that leucocytes and skin (or skin and ovary) share antigens in common, i.e. that at least some antigens present in leucocytes are also present in skin. But we have now shown that, injected at a sufficiently early age, leucocytes and whole blood (of which the leucocyte is the antigenically active element) can confer *tolerance* of skin (§§3·2 to 4, 5, 7), and this leads to an inference of a much more radical kind—that skin epithelium contains no antigens not also present in the leucocytes. For if the leucocyte contained fewer antigens, tolerance would not be conferred in respect of those which were missing, and a skin

homograft transplanted later in life could hardly survive, as it may be seen to do, almost permanently. The same reasoning applies to our demonstration that cells of a mammary carcinoma, inoculated into newborn mice, may confer tolerance of homografts of skin (§9). The wider implications of this lack of tissue specificity in acquired tolerance are discussed below (§11·3).

Individual specificity

Here, too, the induction of tolerance is a much more powerful method of revealing antigenic similarities and differences than the induction of immunity. In a heterogeneous population of rabbits, a homograft from one donor may or may not sensitize its recipient against a homograft transplanted on some later occasion from a different donor (Medawar 1946*a*), and the same is true of mice. If it does so, the second donor may be presumed to possess antigens which were also present in the first; the severity of the cross-reaction depends upon the degree to which the antigenic constitutions of the two donors overlap. But experiments of this kind merely reveal the possession of an indeterminate number of antigens in common, and a strict proof of the specificity of individual antigens had to await Snell's remarkable accomplishment in segregating mouse strains differing by only single 'histocompatibility' alleles (see Snell, Smith & Gabrielson 1953; Snell 1954).

On the other hand, tissues from one donor should confer *tolerance* of tissues taken from another only when the second donor contains *no* antigens not also present in the first. This condition is fulfilled when the donor of the foetal inoculum and the donor of the test graft are members of the same highly inbred strain—the principle upon which we relied in all our experiments on mice (§3·2), and which was denied to us in experiments on other animals (§§3·3, 3·4) by lack of adequately inbred strains. It should also be fulfilled when the first donor is a member of the F_1 hybrid generation of a cross between two inbred animals, and the donor of the test graft is a member of *either* of the two parental strains; for if the two parental inbred strains are truly homozygous, the hybrid progeny of a cross between them must contain at least one representative of every allele present in their parents.

No appreciable tolerance is conferred upon test grafts from a donor which does not fulfil these qualifications. It was shown in §3·3 that 14/17 *WL* chickens injected *in ovo* with the blood of *RIR* embryos were in some degree tolerant of skin homografts transplanted 2 weeks after hatching from their respective donors; seven of the homografts survived for more than 60 days. On six occasions—because the appropriate *RIR* donors had died—the *WL* chicks were grafted with skin from other, randomly chosen, *RIR* donors of the same age. On all six occasions the homografts were destroyed within 9 days. Analysis (§3·3) had already shown that the *RIR* chicks were an antigenically most diverse assembly; there is therefore no reason to doubt that the rejection of test homografts from the new donors was due to their possessing antigens which did not happen to have been present in the donors of the blood used for the embryonic injection.

If the specificity of acquired tolerance is complete, a tolerant adult animal which already carries a normal homograft of long standing should not merely reject a homograft from a new and different donor, but should do so without any deterioration of the homograft already there. We at first believed (Billingham *et al.* 1953), though we later corrected the

mistake (1955), that a tolerated homograft underwent a temporary setback during the rejection of a homograft from a new and different donor, the setback revealing itself by a temporary inflammation and perhaps contracture of the original graft. The mistake was due to using partially tolerant animals; moreover, the setback, though 'immunological', is not of a kind that reflects upon the specificity of acquired tolerance, because it can also occur when the donor of the second test homograft is a member of the same strain as the donor of the first (§4·3). When tolerance is complete, a homograft from a new donor is destroyed without perceptible reaction upon the homograft already present, as the following experiments show.

Two *CBA* mice which had been injected in foetal life with *A*-line cells were grafted with *A*-line skin in the 7th week after birth, and grafted again from the same source 7 weeks later; both the grafts on both the animals remained in perfect condition. When the older homografts were more than 3 months old, the tolerant mice were challenged with skin homografts from *AU* donors. The *AU* homografts were totally destroyed within 10 days; the original, *A*-line, homografts were entirely unaffected (figure 12, plate 7).

A second pair of experiments was performed in the reverse combination. Two *A*-line mice were injected in foetal life with *CBA* tissues, and challenged with *CBA* skin homografts when they were 7 weeks old. The homografts were fully tolerated, and were left undisturbed for 200 days. Both mice were then challenged with homografts from *AU* donors. The newer homografts were destroyed within 10 days; the older homografts from the *CBA* donors were still surviving in a normal condition 120 days later.

In both experiments, therefore, tolerance was strictly specific to donors isogenic with those that provided the cells for the foetal inoculation, and the power of tolerant mice to react upon homografts from unrelated donors was not perceptibly impaired. (Some impairment there must have been, for strains *AU*, *A* and *CBA* share some antigens in common. In the first experiment the effective antigens of the *AU* homografts must have been reduced by the number shared in common with strain *A*; but the residual antigens, those present in *AU* but neither in *CBA* nor *A*, must have been strong enough or numerous enough to provoke an immunity not perceptibly weaker than the normal. Exactly the same reasoning applies to the reaction against *AU* homografts in *A*-line mice made tolerant of homografts from *CBA*.)

Other evidence of the strict specificity of acquired tolerance is referred to in §§6, 8.

4·3. *Adaptation of graft versus adaptation of host*

At first sight it appears self-evident that the adaptation or transformation which makes tolerance possible is an adaptation of the host and not of the graft. The tolerance produced by foetal inoculation is revealed by transplanting a 'test graft' in adult life. The cells of the foetal inoculum could conceivably have undergone some kind of antigenic adaptation, but the test graft, having been freshly transplanted in adult life, could not have done so. As to any adaptation of the cells of the foetal inoculum, Owen's (§2) and our (§7) observations on the persistence of red-cell chimerism after synchorial twinning in cattle and birds seem clearly to belie it. Cells exchanged in foetal life retain the constellation of antigenic properties characteristic of their donors, and it is from these alone that their genetic origins can be inferred. Moreover, the experiments of §6 show that a tolerant host can be caused

to reject a long-tolerated homograft by 'passive' (adoptive) immunization. It follows that the reactivity of the tolerated graft remains unchanged.

Nevertheless, certain evidence of Cannon *et al.* (1954) has been held to point to the occurrence of some measure of adaptation on the part of the grafted cells. Their evidence is of two kinds. In the first place, they showed beyond reasonable doubt that skin homografts transplanted to newborn chicks were more often tolerated when the donors were themselves newborn than when they were as little as 2 weeks old. When the donors were 1 to 7, 12 to 13 and 14 to 16 days old respectively, the numbers of newborn hosts that retained their homografts for 6 months or more were 6/60, 2/62 and 0/82. These findings suggested that the skin from the younger donors, being in some way more 'plastic', was better able to adapt itself to its foreign host.

In the second place, Weber *et al.* (1954) found that an adult chicken, carrying a skin homograft transplanted at its birth, might sometimes reject a second homograft from its original (and now also adult) donor, although the first homograft continued to survive. Five such experiments were described; two were inconclusive, because autografts as well as homografts failed to 'take'; but in two of the other three the second homograft was sloughed away without perceptible injury to the first. This, too, suggested that the first homograft had in some way adapted itself to the foreign soil.

It will now be shown that neither of these two groups of experiments can sustain a hypothesis of adaptation. The stimulus which confers tolerance must be a fully antigenic stimulus (§5). A newborn chick is at the very end of the epoch of life during which it can respond to an antigenic stimulus by becoming tolerant (§4·1). It follows that unless the antigenic stimulus is rather rapidly effective, a state of tolerance will not be induced. The findings of Cannon *et al.* would therefore be completely accounted for if it could be shown that the antigenic stimulus provided by homografts from adult birds were weaker, or slower to take effect, than that arising from the skin of younger birds. So indeed it is. We found that only 1/30 skin homografts transplanted from 2-week-old *RIR* donors to 2-week-old *WL* hosts were still alive 8 days after their transplantation (§3·3); but when the *RIR* donors were adult, 10/18 homografts showed some degree of survival at the 8th day (§5, and table 7*a*, controls). The older skin was therefore *in effect* less antigenic than the younger, presumably because grafts from older animals take longer to establish vascular and lymphatic connexions with their hosts. Although therefore the recipients of younger and older skin in the experiments of Cannon *et al.* were nominally of the same age—newborns—the recipients of the older skin must have been a day or two more advanced in development by the time the antigenic (and therefore tolerance-conferring) stimulus arising from it took effect. Our interpretation of the results of the experiments of Cannon *et al.* is therefore the opposite of their own; it is the younger, not the older skin, that is antigenically the more active, in the sense of being the quicker to exercise its antigenic power.

We may now turn to the problem of the anomalous fate of 'second-stage' homografts described by Weber *et al.* In our experience the rejection of a later homograft by a host which is apparently tolerant of an earlier homograft comes about when, and only when, the state of tolerance is incomplete. In mice we have repeatedly found that when the first homograft is somewhat contracted and scurfy, or not fully haired—outward signs of a real, though feeble, immunological opposition—then the transplantation of a second-stage

homograft may precipitate an immunological crisis as a result of which *both* homografts are eventually destroyed (table 4*B*). If, on the other hand, the immaculate condition of the first homograft reveals a state of complete tolerance, then a second homograft is accepted without perceptible reaction, and both survive (table 4*A*; figure 3, plate 7). A homograft, it should be observed, is likely to be specially vulnerable to opposition during the stage of 'healing in '; although we do not happen to have encountered it in our experiments, it is therefore quite understandable that there should exist a level of immunity at which the

TABLE 4. SURVIVAL TIMES OF SECOND HOMOGRAFTS TRANSPLANTED TO (*A*) FULLY TOLERANT AND (*B*) PARTIALLY TOLERANT MICE AT VARIOUS INTERVALS AFTER THE TRANSPLANTATION OF A FIRST HOMOGRAFT FROM A DONOR OF THE SAME STRAIN (§4·3)

	age of 1st graft at transplantation of 2nd graft (days)	condition of 1st graft	survival time of 2nd graft (days)	comment
(*A*) completely tolerant hosts	25	perfect	>114	both 1st- and 2nd-stage grafts survived until mice were used for other purposes
	25	perfect	>145	
	50	perfect	>39	
	55	perfect	>60	
	77	perfect	>51	
(*B*) partially tolerant hosts	62	contracted	16	1st-stage and 2nd-stage grafts broke down simultaneously
	55	remnant only	16	
	88	poor	40	
	144	poor	24	

TABLE 5. SURVIVAL TIMES OF SECOND HOMOGRAFTS TRANSPLANTED TO FULLY TOLERANT (PARABIOTIC) BIRDS AT VARIOUS INTERVALS AFTER THE TRANSPLANTATION OF FIRST HOMOGRAFTS FROM THEIR RESPECTIVE PARABIOTIC PARTNERS (§4·3)

donor	recipient	age of 1st graft at transplantation of 2nd graft (days)	survival time of 2nd graft (days)	comment
286 (*RIR*)	285 (*WL*)	177	>77	both 1st- and 2nd-stage grafts survived until birds were used for other purposes (see §7·3 and table 9)
285 (*WL*)	286 (*RIR*)	177	>53	
287 (*WL*)	288 (*RIR*)	177	>68	
293 (*WL*)	294 (*RIR*)	148	>67	

second graft succumbs while the first survives. The first homograft has the advantage of a ready-established vascular supply, so that the traumatic inflammation consequent on ordinary healing is not added to any immunological difficulties it may meet. Moreover, the endothelial lining of the blood vessels of the older homograft may have been partly replaced by cells of native origin—an important difference, for it is at the vascular frontiers of a homograft that the reaction against it begins to take effect (Taylor & Lehrfeld 1953).

We thought it proper to confirm this interpretation by experiments on chickens, the subjects of Weber *et al.* own observations. A high degree of tolerance was brought about (see §7) by establishing a parabiotic union between *RIR* and *WL* embryos from the 11th day of embryonic life until hatching; skin grafts were exchanged between the parabionts 2 weeks later. Between 6 and 7 months after hatching, four birds were chosen on which the homografts were in immaculate condition, and these were thereupon grafted, for a second time, with carefully trimmed (§5) skin grafts from their respective parabiotic

partners (table 5). Two of the four second-stage grafts went through a period of vascular dilatation which is not peculiar to homografts, but all four survived until the birds were used for other purposes after 53 to 77 days. It follows that, in birds as in mice (table 4*A*), a fully tolerant host may be repeatedly grafted from its original donor; the case for appeal to a hypothesis of adaptation of the graft does not therefore arise.

There is other evidence that the antigenic constitution of a tolerated graft is not changed by even a long residence on a tolerant host; a tolerated graft (*a*) retains its power to provoke immunity when transplanted into a normal environment, and (*b*) succumbs to the immunity produced by inoculating a tolerant mouse with immunologically active cells. The second phenomenon is discussed in §6. The retention of the power to provoke immunity has been demonstrated on five separate occasions by removing tolerated grafts of 51 to 186 days standing and transplanting them to normal adult mice of the same strain as their former tolerant hosts. The five homografts were duly destroyed, though all outlived their median expectation of survival by 2 to 3 days—a clearly significant prolongation of life. We attribute this to a partial or perhaps complete replacement of the corium and vascular endothelium of the tolerated graft by tissues of host origin; the corium, therefore, would behave as an autograft instead of as a homograft when transplanted from the tolerant host to another member of its strain. The difference may be more important than it appears at first sight, for the reasons mentioned briefly in an earlier paragraph. The 'vascularization' of a normal skin homograft is now known to be brought about very largely by an end-to-end union or apposition of the vessels of graft and bed. In a normal homograft, therefore, the internal blood vessels, which are the first structures to be visibly affected by the homograft reaction, are of foreign origin. On the other hand, the internal blood vessels of a homograft transplanted from a tolerant to a normal host are, in effect, of autogenous origin. Such a homograft is predominantly a homograft of epithelium, and it is known from the experiments of Billingham & Sparrow (1954) on rabbits that a homograft of pure epidermal epithelium survives longer than a homograft of full-thickness skin (13 to 14 days instead of 8 to 9 days).

The acceptance and survival of second-stage homografts in fully tolerant hosts is theoretically of great importance, for reasons quite unconnected with the problems of adaptation. In all our experiments, second-stage homografts have always been transplanted to the side of the body opposite to that occupied by the original test graft. The antigenic matter arising from them must accordingly enter lymph nodes which do not belong to the lymphatic territory of the first-stage grafts (see Billingham, Brent & Medawar 1954). The state of tolerance is therefore 'systemic', i.e. generally in force throughout the body. There is no reason to believe that the original test homograft builds up a privileged position for itself by continuing to bombard its regional nodes with antigenic matter—a position privileged in the sense that tolerance might continue to obtain within its own lymphatic territory when it had disappeared elsewhere. On the contrary, a homograft transplanted to a different lymphatic territory is at no perceptible disadvantage.

In summary, then, tolerance is due not to an adaptation of the graft but to an adaptation of the host. This conclusion is in no way at odds with Koprowski's (1955: see §2) evidence of the antigenic transformation of an ascites tumour growing in mice made tolerant by their injection, as foetuses, with blood. The tolerance so produced is

only partial (§3·2), so that the growth of the tumour was probably opposed by a weak residual immunity. An ascites tumour is essentially a clone of rapidly growing, autonomous and, in some sense, highly mutable cells (see Hauschka 1952). Under such circumstances cellular selection is more than likely; it is almost inevitable. All we have sought to show is that it is neither a normal nor a necessary accompaniment of the adaptation of the host.

4·4. *The secondary response in partial tolerance*

A partially tolerant mouse which has at last thrown off its test homograft may, of course, be grafted for a second time from the same donor, or from a second donor isogenic with the first. When such a second-stage operation is carried out upon normal mice in the

TABLE 6. SECONDARY RESPONSE OF PARTIALLY TOLERANT MICE: THE SURVIVAL TIMES OF SECOND-STAGE HOMOGRAFTS TRANSPLANTED AT VARIOUS INTERVALS AFTER THE BREAKDOWN OF FIRST-STAGE HOMOGRAFTS WAS COMPLETE

All grafts were transplanted in the combinations $A \rightarrow CBA$ or $CBA \rightarrow A$.
For interpretation of results, see text, §4·4.

survival time of first graft (days)	no. of days between breakdown of 1st and transplantation of 2nd graft	survival time of 2nd graft (days)
16	20	7
17	53	10
22	20	17
24	25	8
25	10	9
28	9	13
30	40	11
30	68	14
32	31	13
33	1	9
46	9	12
46	16	8
91*	29	12
93†	9	14
110	18	8

* Third graft transplanted (see text).
† Third and fourth grafts transplanted (see text).

combination $A \rightarrow CBA$, the survival time of the second graft is reduced far beyond its normal value of $11{\cdot}0 \pm 0{\cdot}3$ days (Billingham, Brent & Medawar 1954). The degree of reduction depends upon the time interval between the rejection of the first graft and the transplantation of the second, but the median survival time is not more than 6 days when the interval is as great as 8 months (see §3·2). The secondary response to homografts transplanted in the combination $CBA \rightarrow A$ has not been made the subject of detailed investigation; but, inasmuch as the normal MST is shorter ($10{\cdot}2 \pm 0{\cdot}3$) in this than in the reciprocal transplantation, the secondary response can hardly be less strong.

These data provide the baseline for an investigation of the secondary response in partially tolerant mice, in the combinations $A \rightarrow CBA$ and $CBA \rightarrow A$.

Sixteen partially tolerant mice, which had rejected their first test homografts 16 to 110 (mean 44) days after transplantation, were grafted for a second time, upon the opposite side of the body, 1 to 68 (mean 24) days after the breakdown of the first homografts

(table 6). These sixteen second-stage homografts survived for periods ranging between 7 and 17 days (mean 11·3, standard deviation 2·9, standard error 0·7 day). The second-stage grafts on these partially tolerant mice therefore survived for a much shorter time than their first-stage predecessors; but they survived for about the same length of time, on the average, as first-stage grafts on normal mice. Two of the sixteen mice were grafted for a third time, and one of these for a fourth. Not until the breakdown of the fourth graft was the survival time reduced to so low a value as 6 days, but even here the reaction was decidedly less violent than with second-stage homografts transplanted to normal mice.

These findings show that the state of tolerance represents a *permanent* impairment of the faculty of immunological response. A partially tolerant mouse is not only abnormally unreactive to begin with, but it remains so throughout life. The secondary response is reduced in about the same proportion as the primary response; indeed, the secondary response of a partially tolerant mouse may be no more vigorous than the primary response of a normal mouse.

5. Properties of the stimulus conferring tolerance

5·1. *Preliminary experiments*

In this section it will be shown that the stimulus in response to which embryos or very young animals become tolerant must be an antigenic stimulus, i.e. a stimulus in response to which older or adult animals would have become sensitive or immune. Moreover, the preparations used to confer tolerance upon embryos are antigenic to adults in the same absolute quantities—not merely in the same relative dosages. Five adult *CBA* mice were injected subcutaneously with 10 mm^3 (the standard volume for a foetal injection) of an adult tissue preparation homogeneous with that injected into embryos. Fifteen days later the recipients were tested with skin homografts which were removed for histological examination after a further 6 days. The survival scores of the five homografts extended from 5 % (the survival of the merest trace of homograft epithelium) to 50 % (breakdown in progress but not complete). Homografts on normal mice invariably show 100 % survival at the 6th day (Billingham, Brent, Medawar & Sparrow 1954). The same considerations apply to the injection of embryos with adult blood. The injection of mouse embryos with 10 mm^3 citrated adult whole blood never confers immunity but may sometimes confer tolerance (§3·2). The intravenous or intraperitoneal injection of 10 mm^3 whole blood from *A*- or *CBA*-strain donors into adult *CBA*- or *A*-strain recipients produces a weak but clearly perceptible immunity, breakdown in the majority of the test homografts being complete within 8 days of their transplantation (Billingham, Brent & Medawar unpublished). The comparative weakness of the immunizing power of 10 mm^3 whole blood is doubtless one of the reasons why mouse embryos injected with that quantity become so feebly and irregularly tolerant (see §3·2).

These results show that certain inocula which have the power to confer tolerance are antigenically active, but they establish no necessary causal connexion between the two properties. The experiments now to be described will demonstrate that any one of a variety of treatments which abolish the antigenic power of an inoculum will at the same time abolish its power to confer tolerance.

Preliminary experiments with mice

For the purposes which we had in mind, the results of injecting embryos (whether of mice or birds) were, individually, too unpredictable. Nevertheless, our preliminary experiments with the inoculation of foetal mice were highly indicative. It is well known from the work of Snell and Kaliss (see §11·1) that drying a tissue from the frozen state completely abolishes its power to provoke transplantation immunity—a finding which we have repeatedly confirmed; indeed, for reasons still unknown, the injection of adults with 'lyophilized' tissues actually impairs their powers of immunological response.* We accordingly injected seventy-six foetal mice with an adult tissue preparation identical with that used to confer tolerance (§3·2), except that it had been dried under high vacuum from the frozen state and then reconstituted to its normal fluid volume. Thirty-nine mice survived to be subjected to a test operation 6 to 8 weeks after birth; not one of the thirty-nine homografts was found to have departed significantly from the median expectation of survival on normal mice. The score of 0/39 tolerances may be compared with that which resulted from the injections of foetal mice with living (undried) adult tissues, viz. 63/142 (table 2).

These experiments are nevertheless open to an important objection. Living tissues provide a continuing stimulus, but dried tissues, being dead to begin with, do not. Might not the injection of dried tissues have produced a transient and perhaps feeble state of tolerance, which a test graft transplanted 6 to 8 weeks after injection would not reveal? To meet this objection, and to make the entire test system more predictable and precise, we devised the method now to be described.

5·2. *Test system using newborn chicks*

White Leghorn chicks were injected intravenously, within 12h of hatching, with 0·5ml. whole blood or blood derivatives from adult Rhode Island Red donors. Test grafting from the blood donor was carried out 14 days later, and the fate of the homografts was watched and recorded during the succeeding 30 days. In normal, uninjected chicks (table 7*a*), such homografts do not survive for 20 days, and the great majority are destroyed much sooner. On the other hand, the injection of newly hatched chicks with normal adult whole blood (table 7*b*) or living leucocytes (7*f*) produces a satisfactory proportion of birds in which the homografts are tolerated for *at least* 30 days, viz. $20/46 = 43\frac{1}{2}\%$. It is therefore easily possible to subject whole blood or leucocytes to treatments which are known to destroy their antigenic power to adults, and then to find out if these treatments affect their capacity to produce tolerance after injection into hatchlings.

The advantages of this test system are (*a*) that all the 2-week-old chickens subjected to the test operation were known to have been injected with certainty by a highly effective route, and (*b*) that the test grafting came soon enough after the injection for any feeble or transient state of tolerance to be revealed. It is in no way paradoxical that the intravenous injection of hatchlings with adult blood should give a higher proportion of tolerant birds than grafting at birth with skin (see §§4·1, 4·3), for a skin graft takes several days to become

* Lyophilization does not by any means abolish the power of tissues to elicit the formation of red cell iso-agglutinins; but that is a different matter (Mitchison & Dube 1955).

fully vascular, and its recipient is therefore just so many days older when the stimulus arising from a skin homograft begins to take effect.

Blood was received from a wing vein of its adult donor into a syringe containing 0·1 vol. either of 4% trisodium citrate, or—when the leucocytes were to be handled by procedures involving centrifugation—of a solution of heparin in Ringer's solution containing 100 i.u./ml. Heparinized blood was chilled in siliconed vessels awaiting use. 'Normal whole blood' (table 7*b*) received no other treatment. 'Heated whole blood' (7*c*) is citrated blood which had been heated for 20 min in a thin-walled siliconed test-tube of 100 ml. capacity to a temperature between 49·5 and 50·0° C. 'Plasma' (7*d*) was prepared

TABLE 7. SURVIVAL TIMES OF SKIN HOMOGRAFTS ON TWO-WEEK-OLD CHICKS WHICH HAD BEEN INJECTED AT BIRTH WITH BLOOD AND BLOOD DERIVATIVES TAKEN FROM THEIR FUTURE SKIN DONORS (§5)

Entries in **bold face type** distinguish the injections which conferred tolerance.

expt	substance injected	special treatment	no. of subjects	no. of grafts surviving to 8 days	20 days	30 days	survival to 30 days (%)
a	nil	nil	18	10	0	0	0
b	**whole blood**	**nil**	**28**	**27**	**17**	**13**	**46½**
c	whole blood	heated 49½–50°	16	5	1	0	0
d	plasma	nil	21	11	0	0	0
e	pure red cells	leucocyte-free	18	18	1	0	0
f	**leucocytes**	**nil**	**18**	**18**	**7**	**7**	**39**
g	leucocytes	frozen-thawed	16	10	0	0	0
h	leucocytes	frozen-dried	15	14	0	0	0
i	leucocytes	lysis, bile-salt	13	3	0	0	0
j	leucocytes	ultrasonic (citrate medium)	18	3	0	0	0
k	leucocytes	ultrasonic (Ringer-phosphate)	17	5	0	0	0
totals							
b, *f*	**whole blood and leucocytes**	**nil**	**46**	**45**	**24**	**20**	**43½**
d, *e*	plasma and red cells	leucocyte-free	39	29	1	0	0
c, *f*–*k*	whole blood or leucocytes	destructive treatments	95	40	1	0	0

by the Seitz filtration of the supernatant fluid from strongly centrifuged heparinized whole blood. 'Pure red cells' (7*e*) were also prepared from strongly centrifuged whole blood. The plasma was decanted and the buffy coat lifted off and used for other purposes; the red cells were then resuspended in heparinized Ringer's solution, and passed three times successively through each of two closely packed filter beds of glass wool. (To prepare glass wool of suitable quality, lead-free glass fibre was baked to 250° C for several hours, washed successively with acid, distilled water, alcohol and ether, and dried at 60° C.) For all practical purposes this treatment may be assumed to eliminate white cells completely; only one leucocyte was found in a thick wet film.

Leucocytes (table 7*f*) are less easy to recover from bird's blood than from mammalian blood, for we had no success with attempts to hasten the sedimentation of red cells by adding fibrinogen, according to the technique of Minor & Burnett (§3·2). Instead, heparinized whole blood was subjected to very gentle centrifugation for 30 min. The cells

of the buffy coat were then found to have become sufficiently self-adherent to be lifted off the red-cell sediment with a spatula and a wide-bore pipette. The leucocytes were redispersed in heparinized Ringer's solution and gently spun again. The buffy coat resulting from this second centrifugation was again dispersed in a volume of heparinized Ringer so adjusted as to restore not less than the normal concentration of leucocytes in blood. The great majority, but not all the leucocytes were redispersed as single cells, but some very small agglutinated clumps remained. Red blood corpuscles, though still present, were reduced to about one-fiftieth of their normal concentration.

The leucocytes so prepared were used without further treatment (7*f*), and as the starting point of a number of procedures designed to kill, disintegrate, or otherwise affect the antigenic properties of the injected cells (7*g–k*).

'Frozen-and-thawed leucocytes' (7*g*) are those which had been thrice frozen to −79° C and alternately thawed in a water-bath at 38° C; 'frozen-and-dried leucocytes' were dried for 5 h under high vacuum after freezing the suspension to form a thin shell on the inside of a round-bottomed flask.

The earlier stages of drying were carried out at a temperature below 0° C; in the later stages, the flask was allowed to rise to room temperature. Finally, distilled water was added to restore the original fluid volume. Leucocytes are not disrupted by this treatment, but their cytoplasm becomes curiously sticky and may leave a tail when smeared on glass in making a film.

Leucocytes were disrupted by two methods other than alternate freezing and thawing: by treatment with bile salt (table 7*i*) and by ultrasonic radiation (7*j*, *k*). The former was carried out by mixing 9 ml. of the living leucocyte suspension with 1·0 ml. 0·1 % sodium desoxycholate and incubating the mixture at 38°C for 3 h. All the leucocytes but not quite all the red cells were disrupted by this treatment (it had not been foreseen that so long an exposure to bile salt would be required). Ultrasonic radiation was carried out at a frequency of 300 kc/s with the instrument in the Department of Biophysics in the National Institute for Medical Research; the power transmitted through a crystal transducer in transformer oil was about 10 W/cm^2. The leucocytes were dispersed either in normal citrate saline (0·9 % NaCl 9 vol., 4 % trisodium citrate 1 vol.) (7*j*) or in Ringer phosphate at pH 7·4 (7*k*); they were exposed for three periods of 20 s each in siliconed test-tubes cooled between-whiles to 4° C. The temperature of the oil bath housing the transducer crystal did not exceed 30° C. The anatomical effects of the treatment varied with the nature of the suspending medium. In normal citrate saline the leucocytes were wholly broken up and dispersed as fine particles or fibrous matter. In the presence of calcium, as in Ringer phosphate, the cytoplasm of all the leucocytes was disrupted, but almost all the nuclei remained, in an anatomical sense, intact.

Using the finest hypodermic needle for the purpose, the *WL* chicks were injected within 12 h of hatching through a toe vein or a vein running superficially on the inner aspect of the lower leg. In our experience, these injections are easy and reliable if the leg is held horizontally against a white background, and a hooded lamp, casting no light upwards, is so adjusted as to illuminate the leg from its medial side. Each experiment made use of thirteen to twenty-eight chickens, normally of different hatches; each inoculum was of 0·5 ml. (though 1 ml. is perfectly well tolerated); and all the birds were grafted

with skin from their respective donors 2 weeks later. The skin grafts were disks, approximately 1 cm in diameter, cut from the sparsely feathered skin of the donor's thighs, and transplanted by the method of Cannon & Longmire (1952; see §3·3 and figures 8, 9, plate 7). With adult skin we found it *essential* to trim away all fatty and loose connective tissue matter from the undersides of the grafts before their transplantation. This done, the operations were surgically very successful.

5·3. *Results*

Controls

It has already been shown (§§3·3, 4·1) that 2-week-old chicks are far beyond the stage of development at which the transplantation of a homograft can of itself confer tolerance. Our present results confirm and reinforce this finding. Eighteen untreated 2-week-old *WL* chicks were grafted with skin from adult *RIR* donors by the methods which have just been described. All the homografts were destroyed by the 20th day, though only 10/18 by the 9th day after transplantation (table 7*a*). (These results should be compared with those obtained from grafting 2-week-old recipients with skin from donors which were themselves only 2 weeks old (§3·3): skin homografts from older donors have a somewhat greater median expectation of survival, viz. slightly more as opposed to decidedly less than 8 days following transplantation. The import of the difference is discussed in §4·3.)

The intravenous injection of 0·5 ml. normal adult whole blood conferred tolerance—as measured by a homograft survival time of at least a month—upon $13/28 = 46\frac{1}{2}\%$ of the chicks into which it was injected (table 7*b*). The power to confer tolerance resides entirely in the leucocyte fraction, for living leucocytes (7*f*) were just as effective as whole blood, and plasma (7*d*) and red cells freed from leucocytes (7*e*) were ineffective. Heating whole blood to between 49·5 and 50·0° C for 20 min (7*c*), and all the procedures which killed or disintegrated the leucocytes (7*g–k*), completely abolished the power to confer tolerance. In no case, however, did any of these treatments provoke 'immunity', i.e. cause the test homografts to live for a shorter time than those on the uninjected controls or the recipients of filtered plasma. (The fact that the percentage of homografts surviving to the 8th day was significantly lower in groups 7*i* and 7*j* than in the remainder can be attributed to the use, in these experiments, of 6 to 8-week-old instead of adult birds as donors.)

Extensive observations on the intravenous injection of adult mice with whole blood and its derivatives (to be published in a later paper of this series) shows that the power of blood and its derivatives to incite immunity in adults goes *exactly* hand in hand with its power to confer tolerance on very young animals. The injection of whole blood confers immunity, but its power to do so resides wholly in the leucocyte fraction; plasma and red cells freed from leucocytes are entirely ineffective. All the treatments recorded in table 7, from heating blood to its ultrasonic disintegration, abolish the power of leucocytes to incite immunity;* indeed, in so far as they have any effect at all, it is rather to prolong the life of homografts beyond their normal expectation (see §11·1).

* A reservation should be made in respect of ultrasonic disintegration in a medium which, containing calcium, allows the nuclei of the disrupted cells to remain intact. In the dosages in which they were administered in the present experiments, cells so treated had no perceptible antigenic or tolerance-conferring effect. We wish to leave open the possibility that cell nuclei injected in higher dosages may exert an appreciable antigenic action.

The exact correlation between the power to confer tolerance on very young animals and immunity on older animals justifies the inference that the same properties of the inoculum are responsible for both effects—its antigenic properties. Tolerance is an aberration of the normal mechanism of immunological response brought about by the exposure of immature animals to *antigenic* stimuli, i.e. to stimuli which, had their application been deferred until later in life, would have caused their recipients to have become sensitive or immune.

6. The abolition of tolerance

6·1. *Methods*

A state of tolerance in mice can be permanently abolished by alternative methods, one rapid, and the other slow. If, for example, a tolerant *A*-strain mouse carries a homograft from a donor of strain *CBA*, then tolerance can be abolished, and the tolerated homograft can be caused to slough away, by either of the following means:

(*a*) injecting the tolerant *A* mouse with cells from the regional lymph nodes of normal *A* mice which have been actively immunized by homografts of *CBA*-strain skin;

(*b*) injecting the tolerant *A* mouse with cells from the lymph nodes of normal un-immunized *A* mice.

In general terms, therefore, tolerance may be abolished by the surgical introduction either of immunologically activated or of normal lymph node cells. The former method is of rapid effect; involution of the, until then, tolerated homograft begins within 3 or 4 days of injection and is complete within a maximum of 15 days. The principle upon which it relies is that of 'adoptive immunization' discovered by Mitchison (1953, 1954) and confirmed in detail by ourselves (Billingham, Brent & Medawar 1954): it represents a state of active immunity which has been passively acquired. It will be shown to be *essential* that the donor of either normal or activated lymph nodes should be a member of the same strain as the tolerant mouse into which they are injected, for otherwise the transferred cells would elicit a homograft reaction on their own.

With one minor exception, the subjects of these experiments were tolerant mice carrying skin homografts of upwards of 50 days' standing, the mice being carefully selected for homografts which were normal in every respect. When 'immune' (as opposed to normal) lymph nodes were to be inoculated (table 8*A*), a number of normal mice were immunized by the transplantation of skin grafts taken from members of the donor strain. Ten to twelve days later, when these homografts had duly broken down, a suitable number (eight to fourteen) of regional axillary lymph nodes was excised. In earlier experiments, each node was cut into ten to fifteen pieces with sharp cataract knives, and the assembled fragments (together with matter liberated from them in cutting) were inoculated by trochar into the peritoneal cavity of the tolerant mouse. We later adopted a much more satisfactory method: the lymph nodes were pressed through a fine-meshed stainless steel sieve into Ringer's solution or normal saline. The expressed matter and collected washings were thereupon injected intraperitoneally with an ordinary hypodermic syringe;* the capsular connective tissue was retained by the sieve.

* When the total volume of washings exceeds 2 ml. the cells can be concentrated by light centrifugation. The advice against centrifugation given in our earlier paper (Billingham, Brent & Medawar 1954) was based upon the mistaken use of a citrated suspension fluid.

In experiments making use of 'non-immune' lymph nodes (table 8*B*), fourteen to twenty axillary, cervical and inguinal nodes were collected from a sufficient number of normal, unimmunized donors and treated in a similar way.

In three experiments (table 8*C*) we made use of an immunological variant of the above procedure; ten to sixteen regional axillary lymph nodes were harvested from *AU* mice which had been immunized against *CBA* skin, and were then injected (as a cellular suspension) into *A*-line mice which were fully tolerant of *CBA* skin. In other words, the 'immune lymph nodes' introduced into the tolerant mice were true inter-strain homografts and not, as in the other experiments, the equivalent of autografts. The differences entailed by this procedure are discussed below.

TABLE 8. ABOLITION OF TOLERANCE IN MICE BY THE IMPLANTATION OF 'IMMUNE' (*A*) AND OF NORMAL (*B*) LYMPH NODES, AND FAILURE TO ABOLISH TOLERANCE (*C*) BY IMPLANTING LYMPH NODES FOREIGN TO THE TOLERANT MOUSE'S STRAIN

The mice of groups *A*, *B* were *CBA*'s tolerant of *A*-line tissue, or vice versa; those of group *C* were *A*-line mice, tolerant of *CBA* tissue, and inoculated with nodes from *AU* mice which had been actively immunized against *CBA* tissue. All times are expressed in days. See details in text, §6. See also figures 13 to 15, plate 8.

	age of tolerated graft at implantation of nodes	no. of nodes implanted	form of nodes	time of onset of visible reaction	time of completion of graft breakdown	interval between breakdown of 1st and transplantation of 2nd graft	survival time of 2nd graft
(*A*) immune nodes	109	14	suspension	3	6	18	7
	136	10	suspension	3	9	6	<6
	39	8	fragments	4	11	—	—
	77	8	fragments	3	12	28	8
	101	8	fragments	4	15	4	11
(*B*) normal nodes	120	19	fragments	11	19	2	<6
	57	19	suspension	16	27	17	<8
	144	14	fragments	18	27	—	—
	57	19	suspension	16	34	10	8
	60	20	fragments	28	39	34	9
	57*	19	suspension	44	80	—	—
(*C*) foreign nodes	108	10	suspension	5	recovery by 15	—	—
	138	16	suspension	no reaction	—	—	—
	138	16	suspension	7	recovery by 20	—	—

* Doubtful case: see text.

When the formerly tolerant mice had sloughed away their homografts, the majority were grafted with skin of the same origin for a second time (table 8, last two columns), in order to find out if the immunity set up by the inoculation of lymph nodes continued to be in force.

6·2. *Results*

(*a*) *Inoculation of 'immune' nodes* (table 8*A*; figures 13, 14, plate 8)

Five experiments gave adequately uniform results. Three or four days after the intraperitoneal inoculation of the immune nodes, the homografts became slightly swollen and acquired a persistent dark pink flush. This was succeeded in a few days by hardening, contracture and deterioration of the epithelial surface, and in a few days more by the transformation of the graft into a dry scab which, if not picked off, was undermined and

sloughed away by the ingrowth of the host's epithelium. Breakdown was complete 6 to 15 days after inoculation, i.e. it could be accomplished in a shorter time than the rejection of a freshly transplanted homograft by a normal mouse. Inasmuch as the tolerated homograft had a ready-made blood supply, and was exposed to the force of a ready-made immunity, this is not surprising; but the precipitous course of the breakdown induced by adoptive immunization should be contrasted with the dilatory and, to daily inspection, almost imperceptibly slow deterioration of a homograft undergoing 'spontaneous' retrogression on an incompletely tolerant mouse (§3·2).

(*b*) *Inoculation of 'non-immune' (normal) nodes* (table 8*B*; figures 4, 5, plate 7; figure 15, plate 8)

Six experiments were done. Compared with the effects of implanting 'immune' node cells, the breakdown of a tolerated homograft was slower to begin and somewhat slower in progress; the majority of homografts did not even begin to show signs of retrogression by a time at which, had immune nodes been used, breakdown would have been complete. It may be argued of one mouse (that in which breakdown did not begin until the 44th day and was not complete until the 80th) that retrogression was in reality 'spontaneous' and not the consequence of inoculating it with cells from normal nodes. There is no means of deciding whether or not this objection is valid, but it can hardly apply to the results from the other five. The subjects chosen for these experiments were mice bearing homografts which had been tolerated for 57 to 144 days and which were in immaculate condition when the tests began. With tolerant mice so selected—from which, indeed, these better specimens had been withdrawn—the expectation of 'spontaneous retrogression' is only 25 % in the 50th to 100th day and 11 % in the 100th to 150th day of survival beyond the normal expectation (§3·2).

(*c*) *Inoculation of 'immune nodes' of foreign origin* (table 8*C*)

In these experiments, *A*-line mice fully tolerant of *CBA* skin homografts were inoculated with lymph nodes from *AU* mice which had been actively immunized against *CBA*-line skin. The experiment was therefore identical with that described in paragraph (*a*) except that the immune nodes were foreign to their recipient and must therefore have been destroyed by the transplantation immunity which they themselves provoked.

The results of this experiment were highly revealing. The homografts on two of the three mice (though of more than 100 days' standing) began to deteriorate 5 to 7 days after inoculation, but the deterioration stopped at a stage of mild hyperaemia and slight thickening and contracture, and the grafts returned to a normal state after 15 to 20 days. (Recovery was marked by the prompt onset of a hair growth and pigmentary cycle.) The homograft on the third mouse did not give evidence of even a transient deterioration, and, as it happens, is still surviving at the present time (451 days). Besides adding further weight to the evidence of the specificity of acquired tolerance (§4·2), these results confirm our interpretation of the phenomenon of adoptive immunization, viz. that it is due to the incorporation of living lymph node cells into the mouse into which they are injected, and not to the passive transference of preformed antibody, or a transductive transformation of the host's own lymph node cells (see Billingham, Brent & Medawar 1954). The exact nature

of adoptive immunization is, however, quite irrelevant to the conclusions that will shortly be drawn.

(*d*) *Second-stage grafts*

The survival times of second-stage grafts on tolerant mice which had been caused to reject their first homografts by adoptive immunization are set out in the last column of table 8. All values fall below the median expectation of survival of second-stage homografts in mice which had rejected their tolerated grafts 'spontaneously', and in which immunological reactivity is still subnormal (§4·4, table 6). Tolerant mice injected with normal or activated lymph node cells from normal or immunized donors are thus restored to a state of reactivity. In the long run, there is nothing to choose between the results of implanting immune (table 8*A*) or normal (8*B*) nodes.

6·3. *Conclusions*

The fact that tolerance can be abolished by adoptive immunization in the ways which have just been described makes it possible to draw the following conclusions:

(*a*) Tolerance is due to a *central* failure of the mechanism of immunological response, and not to some intercession with the homograft reaction at a 'peripheral' level (e.g. by absorption of antibodies, or by an amelioration of the inflammatory processes that accompany tissue breakdown). This inference follows directly from the fact that a tolerant mouse is perfectly capable of giving effect to an immune state elicited in a normal animal and then acquired at second hand.

(*b*) The results of the experiments in which tolerant mice were injected with 'immune' nodes shows that the susceptibility of even a long-tolerated homograft to a reaction directed against it remains *completely* unimpaired—clear evidence that an antigenic adaptation of tolerated homografts does not occur (see §4·3).

(*c*) The experiments in which a long-tolerated homograft was caused to undergo regression when its host was inoculated with *normal* node cells carry the argument one stage further. A tolerated homograft does not merely 'contain' antigens; it continues to be a source of antigenic stimuli—or rather, of stimuli which would be antigenic to a host with normal powers of response. The abolition of tolerance by injecting normal lymph node cells can hardly be explained in any other way. These normal lymph node cells are immunologically competent, but not yet active; immunity develops when they respond to the hitherto ineffective antigenic stimuli which must be presumed to issue constantly from the tolerated graft. The wider significance of this fact is discussed below (§11·3). An alternative hypothesis, that the implanted lymph node cells somehow goad a hitherto dormant graft into manufacturing antigens, can hardly be sustained.

(*d*) Adoptive immunization restores a tolerant mouse to a state of normal reactivity.

7. Tolerance, fertility, twinning and red-cell chimerism

7·1. *Relationship between the phenomena*

In dizygotic twin cattle, red-cell chimerism, tolerance of homografts and the infertility of the female member of twin pairs of unlike sex form a well-defined syndrome. It is obvious, moreover, that all dizygotic twin cattle which are red-cell chimeras must be

unable to form iso-agglutinins in response to injections of each other's blood, for the foreign cells introduced by injection would merely be added to those already there. The purpose of this section is to attempt to analyze the causal connexions between these several properties.

It may at once be said that although the correlation between chimerism and infertility in twin cattle is very exact—cows that are not chimeras are not freemartins (Stone *et al.* 1952)—it cannot be regarded as obligatory. Female mice which have been caused to become tolerant by their injection in foetal life with male tissues, including testicular cells, and hens and cocks which have been joined synchorially during the latter half of embryonic life, are without exception fully fertile (Billingham *et al.* 1953; Hašek 1953*b*; and below, §7·3). Presumably the two phenomena go hand in hand in cattle because they have a certain anatomical prerequisite in common, viz. the placental anastomosis which makes possible the exchange of foetal blood.

On the other hand, it is virtually certain that red-cell chimerism must entail a tolerance of skin homografts, for red-cell chimerism comes about when blood cells are exchanged in foetal life, and either adult or foetal blood, when injected intravenously into embryos, is entirely competent to induce a tolerance of later homografts of skin (§§3·2 to 4; 5). There is, of course, no reason why a tolerance of skin homografts produced by the inoculation of tissues lacking red-cell precursors (e.g. tumour fragments, §9) should lead to red-cell chimerism.

So much is straightforward, but the relationship between red-cell chimerism and the inhibition of iso-agglutinin formation in response to cross-transfusions of blood in adult life is not quite so clear. Hašek (1953*b*, 1954; Hašek & Hraba 1955*a*, *b*) found that chickens of different breeds which had been united by a vascular bridge from the 10th day of foetal life until hatching were unable to respond to cross-injections of each other's blood by forming iso-agglutinins in the usual way (Todd 1930). If Hašek's 'parabiotic' birds are in reality red-cell chimeras, the attempt to elicit the formation of iso-agglutinins by cross-injections would be pointless. Hašek & Hraba maintain that their parabionts are *not* chimeras. Our own experiments will now demonstrate that this conclusion is mistaken, but that the chimerism need not be permanent, and that a substantial inhibition of the power to form iso-agglutinins may persist after the state of chimerism has disappeared.

7·2. *Natural and artificial twinning in birds*

Red-cell chimerism is the rule in dizygotic twin cattle (Owen 1945), but in twin sheep (Stormont *et al.* 1953) and twin human beings (Dunsford *et al.* 1953) it is evidently very rare. Chimerism can, of course, only be recognized by immunological methods when the twins are dizygotic and therefore antigenically distinguishable, and it cannot come about unless the twins were synchorial in foetal life.

We find that twin chickens* are normally, perhaps invariably, synchorial. Although an inwardly turned tuck in the chorion marks the frontier between the two sets of membranes,

* I.e. the twins that arise from double-yolked and doubly fertile eggs. The evidence given below shows that such twins are dizygotic, though in an etymological sense they could be described as uniovular. That 'one-yolk' twinning may sometimes occur in birds is apparent from the work of, amongst others, Hess, Juhn & Jull (1947).

there is nevertheless a free anastomosis between the foetal circulations. In each of five separate trials it was found that rabbits' washed red cells introduced into the peripheral blood vessels of one 10- to 11-day-old embryo were found after 3h to be present in roughly equal proportions in its partner's blood.

Presumably, then, twin chicks are red-cell chimeras and are tolerant of homografts of each other's skin. One pair of twin chicks was hatched and reared (a rare occurrence), and 9 days after birth their bloods were tested for intermixture by the method of differential lysis described in full below (§7·3). The results revealed an approximately 50:50 mixture, nine different reagents giving the following percentages of lysis in their respective tubes:

reagent	1	2	3	4	5	6	7	8*A*	8*B*
twin *A*	100	100	50	100	0	0	50	50	100
twin *B*	100	100	50	100	0	0	50	50	100

The cellular residues left after the action of reagents 3, 7, 8*A* (in each case about 50% of the cells originally present) were entirely unaffected by prolonged and repeated exposure to the same reagents freshly administered, but dissolved promptly in reagent 8*B*. There can therefore be no doubt of the existence of a red-cell mixture, and the results prove that the birds were dizygotic twins.

Skin homografts were exchanged between the twins 11 days after hatching, and both survive, in a perfectly normal condition, at the present time (218 days after transplantation). To these results may be added a third—a single twin grafted at birth from its partner, which died in hatching. This homograft still survives after 254 days. (This second result, by itself, is inconclusive, for the reasons given in §4·1.)

All the natural twin chicks we have been able to study were therefore (*a*) synchorial, (*b*) red-cell chimeras, and (*c*) tolerant of grafts of each other's skin. Hašek's technique of vascular anastomosis between chicken embryos has allowed us to extend these results by experimental means.

Hašek's technique consists, essentially, of opposing the bared chorio-allantoic membranes of two chick embryos of 9 to 11 days' incubation, but some kind of bridge is necessary if vascular interpenetration is to occur. To provide such a bridge, Hašek used chick embryos of 24 to 36h incubation; we have found it more convenient to use a thick lens-shaped disk of clotted adult chicken plasma containing coarsely chopped-up fragments of 10- to 11-day embryonic tissue. Two or three drops of heparinized chicken plasma are mixed on a siliconed glass surface with one drop of a dense suspension of chopped-up embryonic heart and skeletal muscle. When clotting has occurred, the firm jelly is prised off with a flexible straight-edged scalpel blade and stored in normal saline until use. As a means of securing vascular union, this method was invariably successful.

White Leghorn and Rhode Island Red embryos of the 10th day of incubation were candled, and suitable areas of the chorio-allantoic membranes near the pointed end of the eggs were marked on the shell. A circular hole 10 to 12 mm in diameter was then cut in each marked area with a mechanical drill; the shell was lifted off and then, with great care, and after wetting with normal saline, the shell membranes were peeled with very fine forceps from the membrane immediately beneath. (For all practical purposes it may be assumed that any puncture of the membrane, however small, is fatal.) The two eggs were

then brought together and manipulated until the windows were almost in apposition; the plasma bridge was then inserted between them, and rotation continued until the two windows came as closely as possible together, edge to edge (figure 16, plate 8). The junction was sealed with paraffin wax thinned with petroleum jelly; each pair of eggs was attached to a separate perforated tray with thin flying buttresses of plaster of Paris (so that the eggs could be turned mechanically in the usual way), and each tray was incubated in a separate compartment of the incubator until hatching.

TABLE 9. SURVIVAL TIMES OF SKIN HOMOGRAFTS EXCHANGED BETWEEN RHODE ISLAND RED AND WHITE LEGHORN CHICKENS WHICH HAD BEEN IN VASCULAR UNION FROM THE 10TH DAY OF EMBRYONIC LIFE UNTIL HATCHING (§7·2)

Except where the contrary is stated, all homografts were transplanted between the chicks two weeks after hatching. See §4·3 and table 5 for 'second-stage' homografts on 285, 286, 288, 294. For tests of red-cell chimerism, see §7·3 and table 10.

no.	breed	sex	survival time (days)	notes
285	*WL*	F	>481	see table 10
286	*RIR*	M	>230*	see table 10
287	*WL*	M	~100	note 'asymmetry'
288	*RIR*	F	>245*	note 'asymmetry'
289	*WL*	F	~200	chimerism lost by 160 days: see table 10
290	*RIR*	M	>163<176	chimerism lost by 160 days: see table 10
291	*WL*	M	>254*	grafted 6 days after birth, on death of 292
292	*RIR*	—	—	grafted 6 days after birth, on death of 292
293	*WL*	M	75	note 'asymmetry'
294	*RIR*	F	>215*	note 'asymmetry'
295	*WL*	—	—	grafted at hatching from 295 (dead in shell)
296	*RIR*	M	>430	grafted at hatching from 295 (dead in shell)

* Grafts in normal condition when birds were destroyed.

The experiments described below were carried out on six parabiotic pairs (of which two were incomplete) which hatched successfully and survived into adult life. Unfortunately, all the subjects of several other operating sessions were lost between the 15th and 17th days of incubation, perhaps because of the harmful influence on the embryos of the adult plasma used to form the vascular bridge (see §3·3). A survival time of 5 to 7 days post-operatively seems to exclude surgical trauma as a cause of death.

Skin grafts were exchanged between the members of the four complete parabiotic twin pairs two weeks after hatching; the two odd twins were grafted at birth and on the 6th day respectively. The survival time of skin homografts exchanged between normal *RIR* and *WL* chicks 2 weeks after birth is less than 9 days (§3·3).

7·3. *Tolerance and chimerism*

The survival times of the homografts exchanged between the members of our six parabiotic pairs are set out in table 9. Tolerance, though variable, was generally very good (figures 17 to 19, plate 8), but the inequalities of survival times between members of individual pairs should not be overlooked (see also Billingham *et al.* 1952). The homografts on animals 286, 288, 291 and 294 were still flourishing when pressure of space, and the completion of the tests described below, obliged us to destroy their hosts after 215 to

254 days. The homografts on 285 and 296 still survive at the present time, i.e. after 481 and 430 days respectively. Homografts on the birds which were grafted 2 weeks after hatching were generally inferior to those on the birds which were grafted at birth (296) or after 6 days (291)—partly because the younger skin used for the grafts contains a higher concentration of feather follicles and so lends itself to a more spectacular result; and partly, perhaps, because the early grafting reinforces, at a critical period, the antigenic stimulus necessary for the induction of complete tolerance.

The four best members of the four complete pairs of parabionts were subjected to a second grafting operation from their respective donors 148 to 177 days after the first; all the second-stage homografts were successful (table 5 and §4·3). As luck had it, all four complete pairs were heterosexual. Both partners of all pairs were fully fertile, as our results with mice had led us to expect (§7·1).

Tests for the presence or absence of red-cell chimerism were carried out on all four complete pairs between the 36th and 47th day after hatching, and again, on two pairs, on the 158th day after hatching. (The lifetime of red cells in adult chickens has been put at about 28 days: Hevesy & Ottesen 1945.) The tests were based upon the use of eight or nine lytic reagents prepared by injecting the blood of chickens into rabbits and absorbing the antiserum so formed with the red cells of single fowls. We are deeply obliged to Dr R. D. Owen for making these reagents available to us, in lyophilized form, and instructing us in their most efficient use. Each test employed the following agents:

(*a*) A 2% suspension in normal saline of the washed corpuscles of each subject.

(*b*) The lytic reagents referred to above, reconstituted to normal serum strength with distilled water and then diluted with three volumes of normal saline—'antibody'.

(*c*) Fresh guinea-pig serum absorbed for 5 min at 0° C with washed, pooled, packed red cells from twelve adult *WL* and *RIR* chickens and then diluted with seven volumes of normal saline—'complement'. All absorptions were complete.

Red cells, antibody and complement were mixed in the proportions 1:2:1 in miniature test-tubes and allowed to stand at room temperature. The tubes were examined and mixed after $\frac{1}{2}$, 2, 4 and, where necessary, 6h.

In the first series of tests, carried out 36 to 47 days after hatching, all eight parabionts gave clear evidence of red-cell mixture; particularly the pair 285/286, where the mixture may have been in the proportion 75:25 in the one bird and 25:75 in the other. Two series of these earlier readings, viz. those that were repeated after 158 days, are cited in table 10. The entry '100' represents complete lysis of all cells; 90 and 75 (figures determined by guesswork and not by counting, for the volumes employed were very small) represent greater or lesser degrees of partial lysis, a well-defined residue of cells being left behind; '+' and 0 represent some detectable lysis and no detectable lysis respectively. In all birds, the results were perfectly complementary; if one tube showed complete lysis, so also did the corresponding tube containing blood cells from the parabiotic partner. It will be seen from table 10 that in the first test on pair 289/290 the readings complementary to 90 are entered as 0. This does not mean that no lysis occurred, but merely that the 10% lysis which might in theory have been expected is too feeble to be seen.

The occurrence of partial lysis in certain tubes was taken as *prima facie* evidence of a genuine mixture of corpuscles, each parabiont containing a small proportion of cells

belonging properly to its partner. This presumption was confirmed in the following way. The unlysed residues left in those tubes in which lysis was still incomplete after 6h were pooled, washed and exposed to a freshly made mixture of the same reagents as before. After 2 to 4h further exposure, the still unlysed residues were again washed, and then exposed to the action of reagents known to dissolve the cells of the parabiotic partner. In the first test on animal 289, for example, the cellular residues unaffected by prolonged and repeated exposure to reagents 5 and 6 were subjected to the action of reagent 7, which had brought about the lysis of 90 % of the cells taken from 290. The cells dissolved within 1h, and sometimes as quickly as within 30 min. The presence of a genuine mixture of corpuscles may therefore be inferred.

TABLE 10. PERSISTENCE OR DISAPPEARANCE OF RED-CELL CHIMERISM IN RHODE ISLAND RED AND WHITE LEGHORN CHICKENS WHICH HAD BEEN IN VASCULAR UNION FROM THE TENTH DAY OF EMBRYONIC LIFE UNTIL HATCHING (§7·3)

First tests were carried out 5 to 7 weeks after hatching; second tests, 5½ months after hatching. At the time of the second tests the grafts exchanged between 285 and 286 were perfect, but those on 289 and 290 were undergoing slow deterioration, complete 3 to 4 weeks later in 290 and 8 weeks later in 289 (table 9). Active cross-immunization (5 weekly injections of 10 ml. whole blood) was begun on completion of the second series of tests.

		lytic reagents									response to active cross-immunization
		1	2	3	4	5	6	7	8*A*	8*B*	
1st test	285	100	100	+	100	+	+	+	+	—	—
	286	100	100	75	100	75	75	75	75	—	—
2nd test	285	—	—	+	—	+	+	+	—	100	nil
	286	—	—	75	—	75	75	75	—	100	nil
1st test	289	100	100	100	100	90	90	0	100	—	—
	290	100	100	100	100	0	0	90	100	—	—
2nd test	289	—	—	—	—	100	100	0	—	—	nil
	290	—	—	—	—	0	0	100	—	—	marginal

100 = rapid and complete lysis of all cells.
75, 90 = greater or lesser residues of cells resistant to lysis.
+ = perceptible lysis.
0 = no perceptible lysis.

The tests were repeated on the pairs 285/6 and 289/290 about 4 months later (table 10, 2nd test). The chimerism of the former pair remained as well defined as formerly, but that of the pair 289/290 had completely disappeared. These results were exactly correlated with the condition of the homografts exchanged between them. When this second test was done, the homografts on 285 and 286 were in perfect condition, and remained so long after the completion of the tests (table 9). On the other hand, the graft from 290 on 289 was seen to have become swollen, featherless and eczematous, with some tendency to local ulceration, by the 144th day after transplantation, and it was still in this condition when the bird was killed on the 200th day. The graft from 289 on 290 had begun to degenerate at the same time, but the deterioration was quicker, and the graft had completely disappeared by the 176th day. In short, chimerism persisted only where the tolerance of a skin homograft was demonstrably still complete.

The loss of chimerism and the deterioration of the homografts in pair 289/290 did not, however, mark a return to normal reactivity. On five weekly occasions, each of the birds

285/286 and 289/290 was injected intramuscularly with 10 ml. of its parabiotic twin's blood. Todd (1930) showed long ago that normal birds of different antigenic constitutions respond to this treatment by the formation of iso-agglutinins. No iso-agglutinins developed in 285/286 (which is hardly surprising, for according to our evidence of chimerism their bloods were already intermixed); none developed in 289, in spite of the fact that its homograft from 290 was deteriorating within this period, and that its chimerism had been lost; and only in 290, a week after the fifth and last injection, was it just possible to detect iso-agglutination, at a titre not exceeding 4, after the agglutination tube had stood for 24h at room temperature. The homograft on this bird had already disappeared.

These results may be thought to clarify the relationship between tolerance of skin homografts, red-cell chimerism, and the inability to form iso-agglutinins in response to the artificial introduction of foreign cells. The state of mutual tolerance brought about by either natural or artificial synchorial twinning of chicken embryos is accompanied by red-cell chimerism; but when tolerance is incomplete, both chimerism and a tolerated homograft will eventually disappear. But the disappearance of tolerance and chimerism does not mark a return to normal reactivity; it merely represents the completion of a long-drawn-out and much-debilitated primary immunological response. The subject remains in a state of enfeebled reactivity, for the secondary response of partially tolerant animals is profoundly impaired (§4·4). The attempt to cross-immunize a pair of ex-chimeras by cross-transfusion is, in effect, an attempt to provoke a secondary immunological response. It is not surprising, then, that the attempt should have failed, as in 289, or should have achieved a merely marginal success, as in 290. In summary, an existing state of red-cell chimerism must of necessity imply a failure to form the appropriate iso-agglutinins, but the disappearance of chimerism does not imply that iso-agglutinins will thereupon be formed. A greater or lesser degree of inhibition will still prevail. Hašek's inference, from his failure to demonstrate chimerism, that chimerism and the inhibition of antibody formation are essentially different phenomena, cannot therefore be upheld.

8. Tolerance of heterologous cells

Hašek's technique of embryonic parabiosis lends itself well to the study of 'heterologous' tolerance in inter-specific, or even in inter-ordinal transplantations, as between chicks and ducks. Skin grafts transplanted from newly hatched chicks to newly hatched ducks are not properly vascularized, and, in default of natural primary healing, can hardly be said to enjoy a 'survival time' at all. In each of fifteen trials, grafts so transplanted were found to have become completely macerated or mummified within 6 days.

This reaction can be profoundly modified. Eighteen day-old duck embryos were parabiotically joined to 11-day chick embryos by the methods already described (§7·2); six ducks were recovered at hatching and grafted at once with skin from the corresponding chicks. The survival times of these six grafts were 8, 8, 20, 25, 27 and 45 days respectively. The grafts were, however, never normal, for even the longest surviving were in a swollen and chronically hyperaemic state (figure 20, plate 8). Heterografts transplanted to three parabiotic ducks from chicks other than their partners were all destroyed within 6 days. The survival times of heterografts in three trials of the converse combination, i.e. from duck to chick, were respectively 10, 15 and 25 days.

In a second test, twelve chick embryos of 10 to 11 days' incubation were each injected intravenously (§3·3) with 0·1 ml. adult duck's blood, and grafted with skin from the adult donor 14 days after hatching: comparison with their ten controls revealed a barely significant prolongation of survival, though the grafts had clearly been vascularized—evidence of some amelioration of the normal response.

In a third test, chicks were injected with 0·1 ml. washed adult duck's blood on the 10th day of incubation and again with 1·0 ml., immediately after hatching. Skin heterografts from the blood donor were transplanted without further delay. Of nine chicks so treated, 4/9 still carried surviving heterologous tissue 10 days after its transplantation; in 1/9 the heterograft survived for 17 days.

The results summarized above show that methods which induce a high degree of tolerance of homologous tissues are only marginally effective when applied to more remotely foreign cells. The most likely explanation of our failure to secure a high degree of tolerance is that exposure to foreign cells by artificial synchorial twinning begins much too late in embryonic life—in chicks at about 10 days. That a high degree of 'heterologous' tolerance can indeed be achieved is implicit in the experimental results of Eastlick (1941), whose donors and recipients were only $2\frac{1}{2}$ to $3\frac{1}{2}$ days old. It is entirely possible (§11·3) that the capacity to respond to antigens of different classes matures at very different epochs of development, and that the inhibition of reactions in which 'natural' antibodies may play a part requires a much earlier and more radical intervention than with forms of immunity which must be actively acquired.

9. Tolerance of tumour homografts

In this section it will be shown that tolerance can be induced by, and in respect of, tumour homografts, and by tumour homografts in respect of skin (see also Koprowski 1955; Bollag 1955). It will also be shown that a degree of tolerance which allows a tumour homograft to flourish may be too low to sustain a homograft of skin; or —to put the same facts in another way—that an immunity strong enough to destroy a skin homograft completely need not stop, though it may temporarily impede, the growth of a homograft of malignant cells.

The tumour used in the experiments now to be described was a mammary adenocarcinoma which arose 'spontaneously' in an adult female *A*-line mouse. Implanted subcutaneously by trochar, this tumour grew progressively in all but one of the *A*-line mice in which it was maintained by serial transfer; in 30/30 adult *CBA* mice it grew to a palpable size and was then resorbed. The experiments made use of the tumour in its first to fifth passages through *A*-line mice.

The tumour was transplanted to thirty-five *CBA* mice within 16 h of birth (cp. Gross §2). For this purpose, it was rendered into a suspension of single cells and minute cell clumps and injected subcutaneously, in a median dorsal position, in volumes of 5 to 20 mm^3 dispensed with a micrometer-controlled syringe. After a variable latent period, sometimes less than 3 weeks, tumours began to grow in 13/35 inoculated mice. (No tumour which grew progressively made its appearance later than after 37 days.) One of these thirteen tumours, after growing to a large size, underwent regression; the remainder grew progressively and, had they been allowed to do so, would have killed their hosts. Taking the

tumour as its own 'test graft', it follows that 37 % of the newborn *CBA* mice were endowed with tolerance of a degree sufficient to allow the tumour homograft to indulge in progressive growth. The comparable figure for tolerance of skin homografts produced in normal mice by the inoculation of normal tissues is $8/94 = 8\frac{1}{2}$ % (table 2, §§3·2, 4·1). The percentage of tumour-tolerant mice might have been higher if it had been feasible to inoculate the newborns with larger fragments instead of with fine suspensions. An inoculum of 20 mm^3 of a preparation homogeneous with that injected into *CBA* newborns gave rise to tumours in only 5/9 adult *A*-line mice; but this may well represent too low an estimate of the efficacy of the cell suspension, for the subcutaneous tissue of adult mice may provide a less suitable environment for survival and growth.

Six progressively growing tumour homografts in the 8th to 14th week of life in their tolerant *CBA* hosts were removed, and clean peripheral fragments were grafted on the one hand into adult *A*-line mice, and on the other hand into normal adult *CBA* mice, to test for any change of antigenic specificity. The grafts grew progressively in 14/15 *A*-line mice and in 0/18 *CBA* mice. It follows that, if such a change occurs at all, it is not the general rule. (A 'solid' tumour, after a single passage through partially tolerant hosts, could hardly be expected to undergo any perceptible selective transformation. It is far otherwise with ascites tumours; see §4·3.)

Between the 34th and 47th day of life, eleven of the thirteen tumour-growing *CBA* mice were grafted with skin from normal *A*-line mice. The fate of the skin homografts was instructive in its wide variety. Three of the eleven homografts broke down before the 8th day, as if their hosts were more than normally resistant; three broke down at the normal time; and the remaining 5/11 exceeded their normal expectation of survival, viz. $11{\cdot}0 \pm 0{\cdot}3$ days (table 1). To only one of the five tolerated skin grafts could a definite survival time be allotted, viz. 16 days; the remainder were still alive when their hosts were killed, in the extreme cases after 56 and 75 days.

These two extreme cases deserve special consideration. In one, the tumour formed a compact subcutaneous growth, 5g in weight, which was surgically excised without recurrence when the skin homograft had been in place for 24 days. The skin homograft survived until the mouse was killed after a further 51 days. The growth of the other tumour was temporarily retarded by the intraperitoneal inoculation on two separate occasions of a total of eighteen regional axillary lymph nodes from normal *CBA* mice which had been actively immunized against *A*-line skin. Such a treatment is normally far more than adequate to abolish a tolerance of skin homografts completely (§6); but on this occasion, the skin homograft was not visibly affected, and the tumour, after its temporary setback, resumed its growth.

It may be assumed without question that the 22/35 *CBA* mice which were *not* tolerant of the *A*-line tumour homografts would not, if tested, have proved to have been tolerant of *A*-line skin. Two tumour-bearing mice which might have been tolerant of skin homografts were not tested, because the size and position of their growths made it impossible to do so. If they were indeed tolerant, then a total of $7/35 = 20$ % of mice inoculated at birth with tumour tissue could be classified as tolerant of skin; if they were not, the proportion would be somewhat lower, viz. $5/35 = 14$ %. Even if the former and more optimistic estimate of 7/35 is accepted, it cannot be inferred that tumour tissue inoculated into

newborn mice is more effective in conferring tolerance of *skin* homografts than inocula of normal adult tissues (8/94, table 2, §4·1; $\chi^2 = 3{\cdot}28$, $P = 0{\cdot}07$). It follows that the occurrence of a genuinely higher proportion of mice which were tolerant of tumour homografts (13/35 as opposed to 8/94; $\chi^2 = 15{\cdot}34$, $P \ll 0{\cdot}01$) is to be attributed to the fact that a very low degree of tolerance may yet suffice to allow a tumour homograft to grow in an alien host (see §11·1).

The tumour tissue was prepared for inoculation into newborn mice in such a way as to ensure that the great majority of the inoculated cells were of tumour epithelium. All organized connective tissue was left behind. It is likely then, if not absolutely certain, that the tolerance which it produced was due to the action of the tumour cells themselves and not to the presence of adventitious leucocytes or stroma. If this is so, the power of an inoculum of tumour tissue to confer tolerance of skin is evidence that skin contains no antigen not also present in the tumour. The reasoning which justifies this inference is given in §4·2. A second inference justified by our findings is that a localized subcutaneous inoculation of foreign cells is competent to bring about a state of tolerance; a systemic dissemination of the inoculated cells, though it may be desirable, is not obligatory. The tumour we worked with did not metastasize; tumours grew only at the site of inoculation.

10. Maternally induced tolerance

The problem of maternally induced tolerance (discussed in general terms in §11·3) may be put in the following way. Might not the accidental incorporation of maternal cells into a mammalian foetus confer upon it some tolerance of homografts of maternal origin? If it did so, homografts transplanted from mothers to offspring should sometimes last much longer than homografts transplanted from the fathers.

Even in cattle, the long gestation period of which should provide a favourable opportunity, such a state of affairs cannot be very common, for on each of eleven occasions skin homografts transplanted from dams to calves of single (i.e. non-twin) birth did not exceed their normal expectation of survival, viz. about 9 days (Billingham *et al.* 1952; Billingham & Lampkin, unpublished). It is true that skin grafts transplanted from dams to calves of dizygotic or multiple twin birth enjoyed an abnormally prolonged survival; but we attributed this to the fact that the twin calves, being tolerant of each other's homografts, must have been unreactive to a much wider range of antigens than a calf of single birth.

Our first experiments were carried out on mice, and their results were reported in the first paper of this series (Billingham, Brent, Medawar & Sparrow 1954). Hybrids of the mating $A \times CBA$ were backcrossed to *A*-line mice in two combinations: $F_1 ♀ \times A ♂$ and $A ♀ \times F_1 ♂$. The offspring of the two reciprocal matings (twenty-five and twenty-three respectively) were challenged in the 6th to 8th week of life with homografts of *CBA*-strain skin. The essential difference between the two classes of R_2 progeny is this: those born of F_1 females underwent gestation within, and were suckled by, animals in which the antigens peculiar to *CBA* mice were necessarily represented; whereas these antigens were absent in the *A*-line mothers of the progeny of the reciprocal backcross. Only the members of the former group, therefore, were exposed to the possible action of *CBA* antigens from conception onwards. In the outcome, however, there was no difference between the survival

times of the *CBA* homografts in the two classes; they extended without distinction from 10 to 19 days.*

A second trial, on a more limited scale, was made with rabbits. Twenty-one rabbits, representing the offspring of four different heterogeneous matings, were grafted when 4 to 5 weeks old with maternal skin. In no rabbit did these homografts survive for more than 12 days, i.e. did they exceed their normal expectation of survival. Here also, then, tolerance of maternal tissue must be unusual, if it occurs at all. The particular interest of these results is that rabbit embryos are known to be infused from an early age with maternal plasma protein (see Brambell, Hemmings & Henderson 1951). Plasma protein is not, of course, iso-antigenic (§5), so no tolerance was to be expected on that account; but our negative findings may be held to indicate that no large number of maternal leucocytes was accidentally incorporated into the foetal tissues.

Rabbits and mice must be regarded as rather unsuitable subjects for these experiments, for if maternally induced tolerance of tissue antigens occurs only by chance, and very infrequently, then the likelihood of its happening should increase with the length of gestation. An experiment on a large scale was therefore carried out on guinea-pigs. Two sets of sixteen litters each were set aside from heterogeneous matings; the male and female parents were chosen for their outward dissimilarity, and normally came from different breeders. The forty-nine members of one set were grafted with skin from their respective mothers, and the forty-seven members of the other set were grafted with skin from their respective fathers. It was for various reasons impracticable, though it would have been desirable, to divide each of the thirty-two litters randomly into maternally and paternally grafted halves; and it would have been unsound to graft each individual with both maternal and paternal skin, for only partial tolerances were expected, and the rejection of (say) a paternal homograft might have curtailed the survival time of the graft transplanted from the mother (see above §§4·2, 4·3).

The guinea-pigs were grafted when they were about a month old. The median survival time of homografts exchanged between genetically diverse adult guinea-pigs is known from the work of Sparrow (1953, 1954) to be about 9 days (see Billingham *et al.* 1954). The survival times of the two groups of homografts in the present experiment are set out in table 11; the litter membership of each of the animals is distinguished by the lettering of the individual entries, but the letters on the left-hand side of the table (maternal homografts) do not correspond with those on the right-hand side (paternal homografts).

The results require little comment. The majority of homografts in both groups broke down before the 12th day, and with the exception of two grafts in the maternal group, the survival times of both sets of grafts were virtually identical. The two exceptional homografts survived for 100 days (after a slow deterioration) and for more than 160 days respectively. The former was carried by an animal whose three litter-mates (*j*) tolerated their homografts for more than 20 and less than 40 days; the latter by an animal three of

* In this genetic situation, the acceptance of a *CBA* homograft by some of the R_2 offspring could be due merely to luck of antigenic segregation; it would be necessary, in the first instance, to demonstrate a clearly significant difference between the percentage of acceptances in the two reciprocal classes and the lengths of time for which the homografts survived. With this in mind, the test as a whole might well be repeated using a more sensitive indicator of very low degrees of tolerance; e.g. by transplanting a tumour indigenous to *CBA* mice to the R_2 offspring at birth (see §9).

whose four litter-mates (*a*) rejected their grafts before 12 days. The graft on the fourth survived for about 35 days.

It is possible that these two exceptionally prolonged survivals were due merely to the luck of antigenic segregation, i.e. to the chance occurrence of two animals containing all, or nearly all, the antigens of their respective mothers. With no knowledge of the number of 'histocompatibility' genes in guinea-pigs, or even of the genetic constitution of the parents, such an interpretation cannot be gainsaid. Given an equal *a priori* likelihood of their turning up in either of the two sets of grafts the odds against the appearance of both anomalies in the maternal moiety are only about three to one. Nevertheless, the quasi-continuous form of the graft mortality distribution suggests that in guinea-pigs, as in mice, the genetic control of histocompatibility is highly multi-factorial; and this, combined with the fact that the two anomalously long-lived homografts form a class standing far apart from the remainder, inclines us to believe that their long survival was due to an actively acquired tolerance of maternal cells.

TABLE 11. SURVIVAL TIMES OF HOMOGRAFTS TRANSPLANTED FROM PARENTS TO OFFSPRING IN GUINEA-PIGS (§10)

Letters indicate litter membership. The animals which received maternal and paternal grafts form independent groups and the lettering on the left does not correspond to the lettering on the right.

maternal grafts	survival time (days)	paternal grafts
oommllkkiiiffddccccbbbaaa	<12	*abbefhhiiiiijjjkkklnnnooopp*
ppnlkkhhhe	13–20	*beeflllp*
njjgfe	21–30	*acdeghmmnp*
onjga	31–40	*dmo*
g	41–50	
	51–60	
	61–70	
	71–80	
	81–90	
j	91–100	
a	$\geqslant 100$	

If tolerance of maternal homografts were regular and frequent, then a state of acquired tolerance could in theory be transmitted from a mother to the succeeding generation. A tolerant mouse is a chimera containing antigenically foreign cells, and these cells, in a female mouse, might so act upon its offspring as to make them tolerant in their turn. Such an effect could only be a weak one, and a direct test gave no grounds for supposing that it occurs at all. A total of twenty-four offspring representing four litters of inbred *CBA* mice born of parents which were both fully tolerant of *A*-line skin were themselves challenged in the 6th week of life with homografts of *A*-line skin. The survival times of the twenty-four homografts were entirely normal; no perceptible tolerance was revealed.

11. DISCUSSION AND INTERPRETATION

11·1. *Definition; related phenomena*

The following conclusions can be drawn from the evidence presented in the body of the text. Tolerance represents the specific (§4·2) and systemic (§4·3) failure of the mechanism of immunological response which is brought about by exposing embryos (§3) or very young

animals (§§4·1, 5, 9) to 'antigenic' stimuli, i.e. to stimuli which would have caused older animals to have become sensitive or immune (§5). Tolerance is due to an adaptation of the reactive mechanism of the host and in no sense to an antigenic adaptation of the tolerated graft (§4·3). More specifically, it is due to a primary central failure of the mechanism of the immunological reaction, and not to some intercession, at a peripheral level, with its power to take effect, for normal reactivity can be established by inoculating tolerant mice with lymph nodes taken from normal members of their own strain (§6). Tolerance exists in every degree (§3·2), but, even when partial, it may nevertheless be permanent, for the secondary response of partially tolerant animals which have rejected their first homografts remains much impaired (§§4·4, 7·3). In the course of normal development, response to an antigen by becoming tolerant gives way to response by becoming sensitive or immune; the transition from the one kind of response to the other occupies a 'null period' during which an antigenic stimulus has no appreciable effect (§4·1).

In so far as it is possible to concentrate this information into a single sentence, tolerance may now be defined as the outcome of an induced specific central failure of the mechanism of immunological response brought about by the exposure of animals to antigenic stimuli before the maturation of the faculty of immunological response. It may be said at once that, so far as present evidence goes, tolerance is merely the outward sign of an absence of immunity. An animal made 'tolerant' of a particular antigen is simply an animal that remains at an embryonic level of reactive competence where that particular antigen is concerned. There is no evidence that tolerance represents a diversion of antibody formation into a new pathway which leads to the formation of an ineffective or inactive antibody, except in so far as 'normal' γ-globulin might be so described.

Before proceeding to a discussion of cognate phenomena, it will be useful to decide what forms of 'tolerance', using that term for the moment in a wider sense, are expressly excluded by the terms of this definition. The acceptance by F_1 hybrid mice of grafts transplanted from members of their parental inbred strains is clearly quite irrelevant, for it is 'natural' and not induced; it is, indeed, merely that special form of compatibility which arises when, although donor and host are by no means antigenically identical, the donor contains no antigens not also present in the host. The prolongation of the life of skin homografts brought about by the administration of cortisone (Billingham, Krohn & Medawar 1951*a*, *b*; Morgan 1951; Sparrow, 1953, 1954) or of trypan blue (Brent unpublished), or by X-irradiation (Dempster, Lennox & Boag 1950)* are all excluded, for all are equally unspecific. For example, X-irradiation reduces a host's power to react against homografts from any donor, and against other antigens, of quite different kinds, as well.

There remain three phenomena which may have something in common with acquired tolerance, in the sense that they may achieve the same end-result by different means.

The first is the phenomenon of 'immunological paralysis' described by Felton (1949) (see also Felton, Kauffmann, Prescott & Ottinger 1955). Felton showed that mice could be protected against an otherwise lethal dose of living pneumococci by the injection of 0·0005 mg type-specific pneumococcal polysaccharide 7 days beforehand. A larger dose, 0·5 mg, had a 'paralyzing' action; the mice so treated were vulnerable to living pneumococci,

* For a general summary of these phenomena, see Toolan (1953).

and could no longer be protected by the injection, on a later occasion, of the smaller and otherwise effective dose. Although there can be no doubt that the pneumococcal polysaccharides are 'complete' antigens—doubts which have arisen in the past about their antigenic properties may have been caused by the inadvertent use of paralyzing doses (Morgan, Watson & Cromartie 1952)—their long, perhaps indefinite persistence in the tissues may be a genuine peculiarity. Until immunological paralysis has been investigated by the technique of 'adoptive immunization' (§6) it would be wise to classify it as a phenomenon apart.

A second phenomenon is the inhibition of 'drug allergy' described by Sulzberger (1939) and by Chase (1946). Sulzberger found that the injection of arsphenamine into the blood stream protected guinea-pigs from its sensitizing action when applied, simultaneously or later, to the skin. Chase discovered that feeding guinea-pigs by mouth with an oily solution of dinitrochlorobenzene had the same effect. The inhibition was long-lasting and specific; sensitivity to, for example, *o*-chlorobenzoyl chloride, was unimpaired. The inhibition was, moreover, central and not peripheral (Chase 1949), for blocking or inhibitory antibodies could not be demonstrated, and normal reactivity could be restored by injecting cells from the peritoneal exudates of actively immunized guinea-pigs. (It was this ingenious and telling experiment that led us to our own; see §6.) It follows that the experimental inhibition of drug allergy is much the same as tolerance in its final outcome, though it is produced by entirely different means.

Much the same may be said of the abrogation of immunity to tumour homografts by the injection of their intended hosts with a variety of 'devitalized' tissues—a phenomenon of which Snell and Kaliss have made a particularly thorough study (Snell, Cloudman, Failor & Douglass 1946; Kaliss & Snell 1951; Snell 1952, 1954; Kaliss 1955). Here, too, the inhibition of response is specific and long lasting; it is ineffective when the hosts are already immune; and, as Mitchison discovered (see Kaliss 1955), normal resistance is promptly restored by adoptive immunization—brought about, as in our own experiments, by inoculating the treated mice with cells from the regional lymph nodes of actively immunized mice belonging to the same inbred strain. To anticipate evidence that will be given in a later paper of this series, it should be said at once that the weakening of immunity produced by the injection of mice with lyophilized tissues is, quantitatively, of a rather minor character. The effect of five weekly injections of 50 mg dry weight of lyophilized donor tissue is only such as to raise the median survival time of skin homografts from about 10 to about 20 days. In other words, the susceptibility of treated mice to transplanted tumours gives a greatly exaggerated estimate of the degree of inhibition which has been achieved. Immunity is only so far impaired as to allow the tumour to gain a foothold and to grow to such a size and at such a rate that its further progress cannot be effectively opposed (§9).

The properties that tolerance and 'enhancement' possess in common are those which follow from the fact that both represent the outcome of specific central failures of response. But the means by which they are achieved, and in all probability their mechanisms, are so different as to make one cautious of describing them, as Snell (1954) has proposed, by the same term. The stimulus which confers tolerance upon embryos merely incites immunity in adults; the stimulus which enhances the growth of homografts in adults does not pre-

judice the normal differentiation of immature antibody-forming cells (§5). The one represents the effect of a complete antigen on an immature subject; the other, the effect of a modified antigenic stimulus on a system which is fully capable of an immune response. For the time being, perhaps, the distinction should be made evident by the use of different terms.

It should be added that the life of skin homografts in rabbits may be prolonged threefold or fourfold by the intravenous injection of their recipient with a suspension of living epidermal cells, or even with whole blood, taken from the future skin donor (Billingham & Sparrow 1955). It remains to be seen whether this is a phenomenon *sui generis* or a variant of that described above.

11·2. *Tolerance in normal development*

Knowing only of the work of Owen (1945) beforehand, Burnet & Fenner (1949) were led to predict the phenomenon of tolerance on the grounds that antibody-forming cells, characteristically scavengers, must 'learn' to distinguish between substances which are proper to the individual and those which may later gain entry from outside. (Their prediction was founded upon the theory that antibody formation represents an adaptive, heritable cellular transformation, akin to, but not identical with, that which allows bacterial clones to become adapted to the use of new substrates, or to resist the action of inhibitory drugs.)

We believe that Burnet & Fenner's argument applies with particular force to the specialized and complex substances that are formed in the later stages of cellular differentiation. Many such substances might be antigenic, were it not for the fact that the future antibody-forming system is exposed to their influence at a sufficiently early stage. The formation of iso-antibodies in response to the injection of lens protein (or certain ingredients of brain tissue) is at once intelligible in the light of this theory, for at no stage of development should lens protein normally gain access to a seat of immunological response. The same principle applies, for somewhat different reasons, to the formation of iso-antibodies against spermatozoa and casein, for neither develop until the antibody-forming system is mature.* There may well exist a wide variety of bodily constituents which are potentially auto-antigenic, and therefore iso-antigenic, simply because the antibody-forming system has no normal opportunity to become tolerant of their action.

The iso-antigens that are responsible for transplantation immunity do not, however, fall into this category. They are almost certainly represented in every living nucleated cell (§4·2 and below, §11·3), including, therefore, the very cells that are responsible for immunological responses. A future antibody-forming cell need not learn not to react against substances which are part of its own fabric. This reservation does not in any way derogate from the importance of Burnet & Fenner's hypothesis. It remains true that learning not to react against the specialized end-products of cellular differentiation is a prudent safeguard, and that artificially acquired tolerance owes its success to taking advantage of antibody-forming cells when they are still at an educable stage.

If the sharp distinction we have drawn between two distinct classes of iso-antigens is correct, it follows that the cellular substances which act as iso-antigens in transplantation

* The iso-antigenic action of bodily constituents is discussed by Landsteiner (1945, p. 103); see also Freund, Lipton & Thompson (1953) and Boyd (1954).

immunity could not, under any circumstances, be auto-antigenic, i.e. they could not elicit an immune response from the organism of which they form a part. On the other hand, lens protein and spermatozoa should be iso-antigenic only because they are potentially auto-antigenic, and they are potentially auto-antigenic because their owner's antibody-forming cells have no opportunity to become tolerant of their action.

11·3. *Special implications*

Four special implications of the phenomenon of tolerance will now be singled out for discussion under the following headings: (*a*) the immunological relationship between mother and foetus; (*b*) the maturation of the antibody-forming system; (*c*) the antigenic composition of the tissues of the individual; and (*d*) the normal fate of iso-antigens.

(*a*) *The immunological relationship between mother and foetus*

This relationship, in mammals, is usually thought to be one in which the foetus plays the part of antigen. The phenomenon of tolerance (see §10) shows that the relationship can be read the other way about, with the mother as a potential source (or route of access) of antigenic matter, and the foetus as the victim or beneficiary of any state of tolerance which the maternal antigens might induce.

It occurred independently to Professor F. W. Rogers Brambell, Dr N. A. Mitchison, and Dr R. D. Owen that if, in human beings, *Rh* antigens were to enter a *Rh*-negative foetus, they might lower, if not abolish, its power to react against *Rh* antigens in later life. Owen and his colleagues (Owen, Wood, Foord, Sturgeon & Baldwin 1954) have found that sensitization to *Rh* antigens is significantly less frequent in women born of *Rh*-positive than in women born of *Rh*-negative mothers. The degree of sensitization (and so, inversely, of tolerance) was measured serologically. On medical grounds it is unfortunate that neither this nor an earlier study (Booth, Dunsford, Grant & Murray 1953) gave any indication that the *Rh*-positive children of this relatively 'tolerant' group of mothers were any the less likely to be victims of haemolytic disease.

Our own evidence, it will be recalled (§10), is that a naturally acquired tolerance of maternal homografts occurs rarely, if at all; but although the results were not fully decisive, there were good grounds for believing that tolerance of maternal homografts might occur accidentally in guinea-pigs. Certain clinical evidence also suggests that something of the kind may happen. There is no reason to suppose that spontaneous tumours are more easily transplantable between two human beings than between two mice or rabbits of different strains or breeds; yet examples are known in which melanomatous tumour cells of maternal origin appear to have passed the placental barrier and established themselves in the tissues of the child (Weber, Schwartz & Hellenschmied 1930; Holland 1933; Dargeon, Eversole & del Luca 1950; review by Wells 1940). In an immunological sense their behaviour may be exactly analogous to the growth of tumour homografts after transplantation to newborn mice (§9).

In theory, tolerance may be conferred upon a foetus not merely by maternal cellular antigens, but by any foreign antigen (e.g. of bacterial origin) which the mother might transmit passively to the child. This possibility should be kept in mind when interpreting a 'family history' of susceptibility to infectious disease. 'Genetic predisposition' is not the

only possible explanation of a tendency for certain infectious diseases to run in families (cp. the experiments of Traub referred to in §2). There are, in short, two excellent biological reasons why the mammalian foetus should be strictly isolated from the mother at a vascular level. One, well known, is that the foetus should not immunize the mother; a second is that antigens gaining entry through the mother should not impair the immunological responses of the child.

(*b*) *The maturation of the antibody-forming system*

The stage of development at which response by tolerance gives way to response in the adult fashion, by immunity, may be expected to vary from species to species in accordance with the degree of maturity at term; and it is obvious that, by one means or another, an animal must remain under a protective screen of maternal antibodies until its own faculties mature. Birth itself is no guide to the time of origin of immunological maturity. A certain small proportion of newborn mice (§4·1) and newly hatched chicks (§5) are still capable of becoming tolerant in response to iso-antigenic stimulation, but with ungulates such as sheep and cattle the epoch of embryonic reactivity ends long before birth. A skin homograft reaction is well developed in foetal sheep of 100 (out of 150) days' gestation (Schinkel & Ferguson 1953), and is little, if at all, less well developed in newborn calves than in cattle 4 or 5 weeks old (Lampkin 1955). On the other hand, calves up to 4 weeks old produce no detectable antibody in response to intramuscular injections of *Trichomonas foetus* antigen (Kerr & Robertson 1954); indeed, if the doses are large enough, their power to do so in later life is seriously impaired (see §2). Yet at this age the power to react upon and reject skin homografts is fully developed (Anderson *et al.* 1951; Billingham *et al.* 1952).*

These are important facts because they show that, in any one individual, the power to react against different antigens may mature at very different times; it does not develop as an embryological entity. A comparison between the times of onset of transplantation immunity and of immunity to bacterial antigens is clearly valid when an all-or-nothing distinction can be drawn—when, as in the example given above, tolerance of one antigen may still come about at a stage when a different antigen may provoke immunity. Quantitative distinctions are much less certain, but the evidence, so far as it goes, suggests that immunity to foreign proteins is slower to develop in both chicks (cf. Wolfe & Dilks 1948) and rabbits (Baumgartner 1937). A similar distinction of tempo appears in a comparison between immunity to homografts and to heterografts. Our own failure to obtain more than a minor and transient tolerance of chick heterografts on ducks (and vice versa)

* Experiments which are still in progress have already shown that, even with iso-antigens, the end of the epoch of development during which it is possible to induce tolerance varies from one antigen to another: the epoch ends earlier for the antigens which are the more 'foreign' to the animals into which they are introduced. When foetal *A*-line mice were injected with a *mixture* of *CBA* and *AU* cells, and were tested later in life with skin homografts from both donor strains, the *CBA* graft was tolerated more often than the *AU* graft. The evidence of Billingham, Brent and Medawar (1954) shows that, in an immunological sense, the *CBA* donors are the more closely related to strain *A*. Again, when newly hatched White Leghorn chicks were injected intravenously with 1 ml. of an equal mixture of bloods from adult Rhode Island Red and Black Leghorn donors, and were tested with skin homografts from both donors two weeks later, it was found that 15/25 birds carried fully surviving *RIR* grafts after 30 days. Only 10/25 were equally tolerant of *BL* grafts; 5/25 accepted *RIR* but rejected *BL* grafts. A survival-time analysis showed that, of the two donors, the Black Leghorn was the more foreign to the recipient strain.

was almost certainly due to the fact that the chick and duck embryos were already much too old when they were synchorially united or when they received their first exposure to heterologous cells (see §8). (As we have already pointed out, it is implicit in the work of Eastlick that a high degree of heterologous tolerance can be achieved, provided only that exposure to foreign cells occurs at a sufficiently early stage.) The interpretation is complicated, however, by the occurrence of 'natural' antibodies.

The fact that foetal sheep can react against homografts is particularly informative because, like other foetal ungulates,* they are natural 'agammaglobulinaemics'; their γ-globulins, together with antibodies, are acquired at birth (Moore & McCarthy 1946; Charlwood & Thomson 1948). Only later do they make their own. That pathologically 'agammaglobulinaemic' human beings may be unable to react against homografts (Good & Varco 1955) must not be construed as evidence that the homograft reaction is mediated by 'orthodox' antibodies belonging to the γ-globulin fraction of serum protein. It simply indicates that the abnormality which has agammaglobulinaemia as one of its consequences has the inability to react against homografts as a second consequence. Quite different cellular mechanisms may be involved.

(c) The antigenic composition of the tissues of the individual

Special attention has already been called to the fact that the induction of tolerance is not specific to the tissues of an individual: injections of leucocytes or of mammary tumour cells may confer tolerance of later homografts of skin (§§4·2, 9). It follows, then, that skin contains no antigens† that are not also present in the leucocyte or the cells of a mammary tumour (see §4·2); and from this fact two important inferences, one practical and the other theoretical, may be drawn.

The practical inference is that any attempt to subdue or abolish the immunological response of adult animals (including human beings) to tissue homografts—whether by 'desensitization' or by the injection of modified antigens (§11·1)—may be founded upon the use of the donor's blood, i.e. of the tissue which is easiest to come by and easiest to spare. The attempt to make skin homografts acceptable to adult animals by injecting them beforehand with skin extractives (e.g. Allen, Williams, Lovingood & Ellison 1952; Hardin & Werner 1955) depends upon an assumption for which, luckily, there is no evidence whatsoever, namely, that the cells of skin contain 'transplantation' iso-antigens peculiar to themselves.

The second inference owes its significance to the fact that the antigenic composition of cells provides the most immediate and the most comprehensive single measurement of their genetic difference or uniformity. The absence of tissue specificity in tolerance is therefore consistent with the belief that somatic tissues of very different types and of very distant common ancestry in development have similar genetic constitutions. Modern embryological evidence is consistent with this view (cf. King & Briggs 1954). It is not, however, universally valid. Red cells lack the antigens which are responsible for tissue

* See Brambell, Hemmings & Henderson (1951, p. 33).

† I.e. no iso-antigens responsible for the phenomenon of tissue transplantation immunity. That the tissues of a single individual may differ in respect of the antigens that may be revealed by 'heterologous' immunity reactions has long been known; see also §11·2.

transplantation immunity; their injection produces neither immunity against nor tolerance of homografts of skin (§5).* Other tissues or cells (e.g. lens, spermatozoa) contain specialized products of differentiation which may be iso-antigenic because the antibody-forming system has no normal opportunity to become tolerant of their action (see §11·2). The wide distribution of true transplantation antigens in different somatic tissues may be taken as evidence that, as a class, they have a more general and proximate status in the structure and activity of the cell.

It will be noticed that neither of these inferences could have been drawn from investigations on acquired immunity. That the injection of leucocytes will immunize an adult animal against homografts of skin (Medawar 1946*b*) is evidence that leucocytes and skin epithelium share antigens in common. It would be most surprising if they did not. But that leucocytes, injected under the appropriate conditions, will confer *tolerance* of homografts of skin justifies the much more radical inference that was drawn above, namely, that every antigen in skin is also represented in the leucocyte.

(d) The normal fate of iso-antigens

No individual iso-antigen concerned with transplantation immunity is in itself an indispensable substance; an iso-antigen cannot be recognized unless there exists at least one individual in which it does not occur and to which it is therefore inessential. The combinational variety of iso-antigens may be to some extent accidental or capricious; but, for the reasons given in the preceding paragraphs, they must be assumed to be variants of a class of substances which serve some unspecialized metabolic function in the cell.

Genetical analysis (reviewed by Little, its pioneer, in 1941) shows that mouse strains of distant common ancestry may differ by upwards of a dozen iso-antigens that control the fate of homografts of normal cells; rabbits of a heterogeneous population have been proved by a form of combinational analysis to differ by at least seven such antigens, and, in all probability, by many more (Medawar 1945). When tissues are grafted from one individual to another, it follows that quite a battery of complex cellular substances must leave the grafts, enter the lymphatics, and be transported to the spleen and regional nodes (Mitchison 1953, 1954; Billingham, Brent & Medawar 1954). (Mitchison's work proved for the first time that the reaction against a homograft is a true physiological *actio in distans*; it is not undertaken by the tissues in the immediate neighbourhood of the graft.)

A study of the abolition of tolerance (§6) shows that the constant manufacture and emission of complex matter is a normal activity of cells. For tolerance in mice can be abolished in alternative ways: (*a*) by inoculating a tolerant mouse with cells taken from the lymph nodes of an animal actively immunized against the tolerated antigens, and (*b*) by inoculating a tolerant mouse with cells from normal (i.e. unactivated, but immunologically competent) nodes. The rapid destruction of a homograft that is brought about by the former of these methods need not be held to depend upon the emergence of antigenic matter from the graft, for the state of immunity is ready made and has merely to take

* It would be profoundly interesting to know if the foetal injection of red cells freed from leucocytes could reduce or abolish the formation of iso-agglutinins in later life. If it did so, the case for drawing a sharp distinction between transplantation immunity and the formation of red-cell iso-agglutinins would be complete.

effect. But the regression of a homograft brought about by the second method can only be due to an active immunization of the normal node cells by antigenic matter issuing from the tolerated foreign tissue. The antigenic matter may come from the tolerated graft itself, or it may come in part from the surviving residues of the foetal inoculum, but the same principle applies to both. The grafts are a continuing source of would-be antigenic stimuli which exert no effect in a tolerant mouse until it is surgically endowed with cells which are competent to respond.

This inference, obvious enough in itself, owes its interest to the fact that the antigenic matter is highly diverse and complex; it is presumably contained within cellular fragments or sheddings, or perhaps within organized intracellular particles. The constant emission of such substances by normal cells, and their clearance via the lymphatics, is a physiological transaction of a hitherto unrecognized character, and one which only the use of iso-antigenic labelling has so far served to reveal. Its function, if not merely excretory, is quite obscure.

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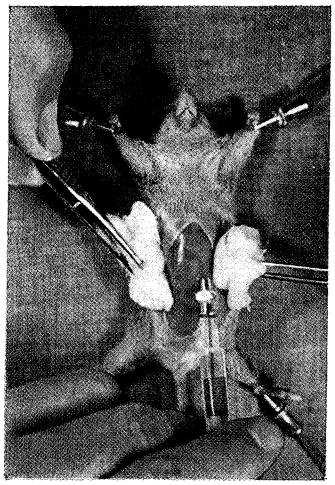

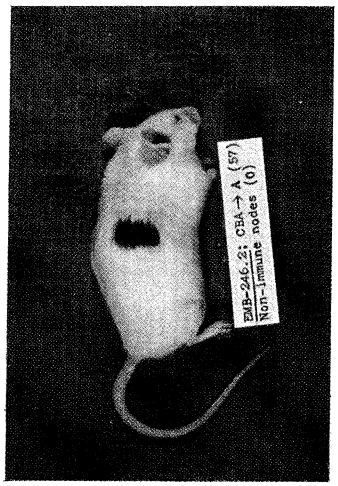

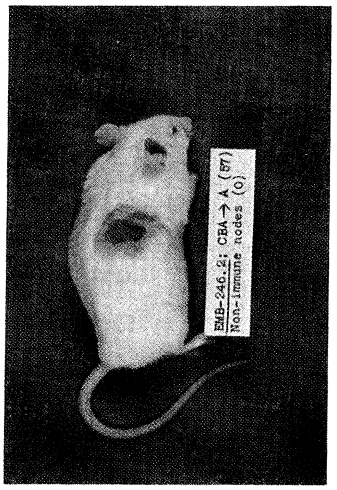

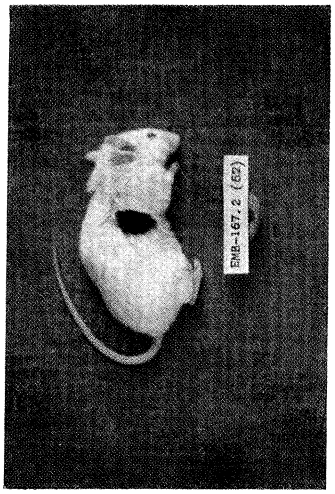

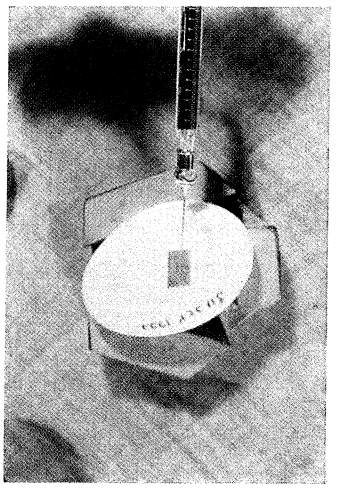

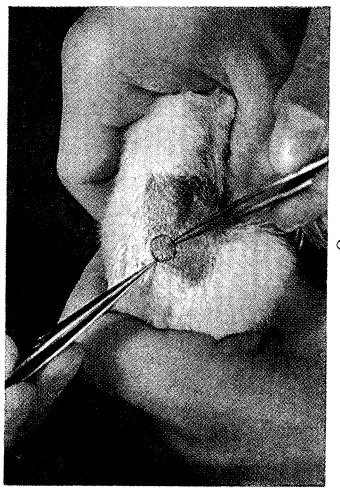

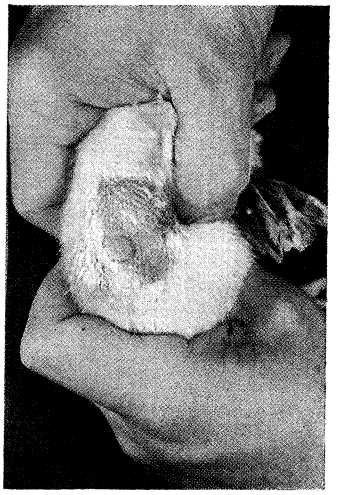

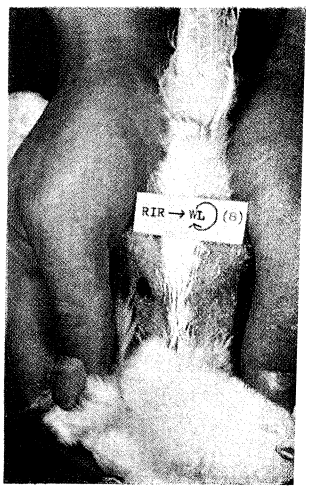

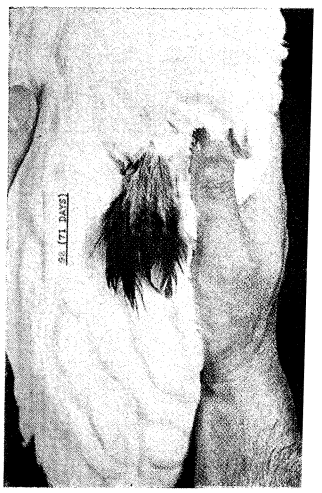

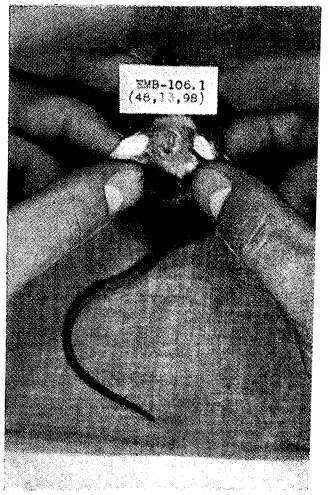

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PLATE 7

FIGURE 1. The technique of injecting mouse embryos through the body wall without laparotomy. The skin has been opened down the ventral midline and gently parted; 17-day embryos have been brought into view and are being injected with a suspension of adult tissues through a fine needle attached to a micrometer-controlled syringe (§3·2.)

FIGURE 2. The appearance of an *A*-line skin homograft 11 days after transplantation to a normal adult *CBA* mouse. Breakdown is complete, and the graft is drying in air to form a scab. Contrast figures 3 to 6. (§3·2.)

FIGURE 3. *A*-line skin homografts on a group of tolerant adult *CBA* mice belonging to a single litter, the members of which had been injected *in utero* with an adult *A*-line tissue suspension. Each mouse bears two homografts, that on the right transplanted 83 days beforehand, that on the left 50 days later. The grafts are perfectly normal. Contrast figure 2. (§3·2.)

FIGURES 4, 5. A *CBA* skin homograft 57 days after transplantation to a tolerant adult *A*-line mouse which had been injected during uterine life with an adult *CBA* tissue suspension. The graft is shown before (figure 4) and after (figure 5) clipping away its pelt of normal agouti hairs: it has been fully incorporated into the skin of its host. (For the later history of this graft, refer to figure 15.) (§3·2.)

FIGURE 6. An *AU* (black-haired) skin homograft 52 days after transplantation to an *A*-line mouse which had been injected *in utero* with whole blood from an adult *AU* donor. (§3·2.)

FIGURE 7. The technique of intravenous injection of chicken embryos. A rectangle of shell lying over a prominent chorio-allantoic vein has been removed and the shell membranes have been made transparent by the application of paraffin oil. A fine needle mounted on a tuberculin syringe has been inserted coaxially with the vein and in the direction of blood flow. For the tolerance induced by this procedure, see figure 11. (§3·3.)

FIGURES 8, 9. The 'test operation' in two-week-old chicks: a skin graft is being put into place on a muscular bed lying dorso-laterally and anteriorly to the sacrum (figure 8); it is then held in place by a film of plasticized collodion applied in solution and allowed to dry (Cannon & Longmire 1952). (§3·3.)

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FIGURE 11. A skin homograft 71 days after transplantation from a 2-week-old Rhode Island Red chick to a 2-week-old White Leghorn chick which had been injected with 0·2 ml. of its future skin donor's blood on the 11th day of embryonic life. The graft has differentiated normally. (§3·3.)

FIGURE 12. The specificity of acquired tolerance. A *CBA* mouse, tolerant of *A*-line tissues, carries two *A*-line homografts, of 48 and of 98 days' standing respectively. Thirteen days before this photograph was taken, a skin homograft from an *AU* donor had been transplanted between the two grafts already in place. The *AU* homograft is totally destroyed. (§4·2.)

PLATE 8

FIGURES 13, 14. Abolition of tolerance by the implantation of 'immune' node cells. Seventy-seven days after the transplantation of an *A*-line skin homograft to a fully tolerant *CBA* host, the host was injected intraperitoneally with cells expressed from the regional lymph nodes of normal *CBA* mice which had been actively immunized against *A*-line skin. The hitherto tolerated *A*-line homograft became grossly inflamed within 8 days of the inoculation (figure 13) and its breakdown was complete in 12 days (figure 14). Contrast figure 15. (Table 8*A* and §6).

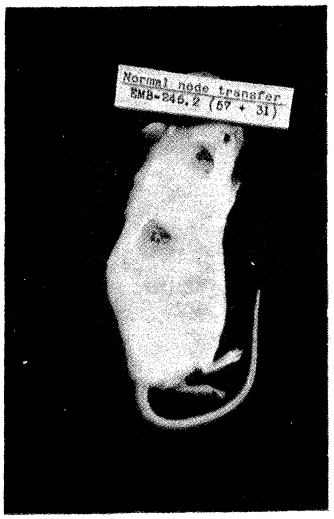
FIGURE 15. Abolition of tolerance by the implantation of normal nodes (contrast figures 13, 14). The tolerant *A*-line mouse illustrated in figures 4 and 5, carrying a *CBA* homograft of 57 days' standing, was inoculated with cells expressed from the lymph nodes of normal *A*-line mice. The present photograph illustrates the appearance of the homograft 31 days later: total breakdown. (Table 8*B* and §6.)

FIGURE 16. The technique of artificial synchorial twinning ('parabiosis') in 10- to 11-day-old chicken embryos. The shell and shell membranes have been removed over a circular area in both eggs to expose the chorio-allantoic membranes. The bared areas are being brought face to face, and a plasma bridge is being inserted between them to make a vascular connexion. (§7.)

FIGURE 17. Illustrating the perfectly normal differentiation of a White Leghorn skin homograft 26 days after transplantation to a Rhode Island Red host with which the graft donor had been in parabiotic union. The graft has healed soundly and White Leghorn feathers are beginning to grow. Contrast the heterograft illustrated by figure 20. (§7.)

FIGURES 18, 19. Homografts on birds which had been synchorially united to their future donors during embryonic life. Figure 18 shows a Rhode Island Red graft 282 days after transplantation to its 6-day-old White Leghorn parabiotic partner; figure 19 shows a White Leghorn graft 240 days after transplantation to its newly hatched Rhode Island Red parabiotic partner. (Table 9, §7.)

FIGURE 20. A chicken skin heterograft 26 days after transplantation to a newly hatched duck with which it had been in synchorial union during the latter half of embryonic life. Contrast figure 17. The heterograft survives, but it is swollen and inflamed, and imperfectly differentiated. (§8).



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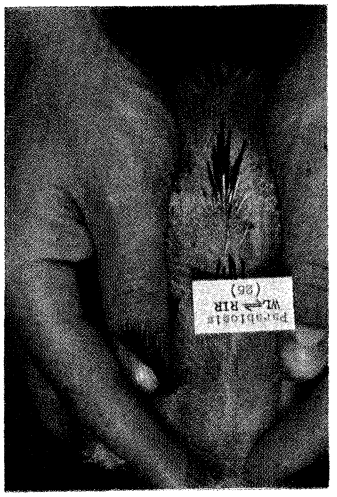

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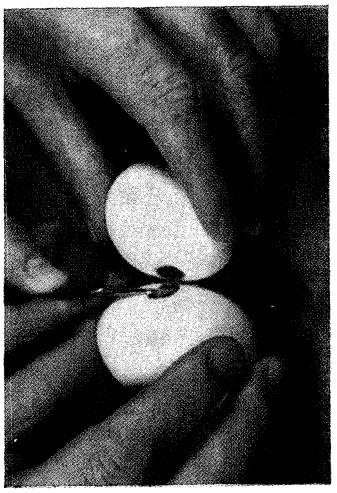

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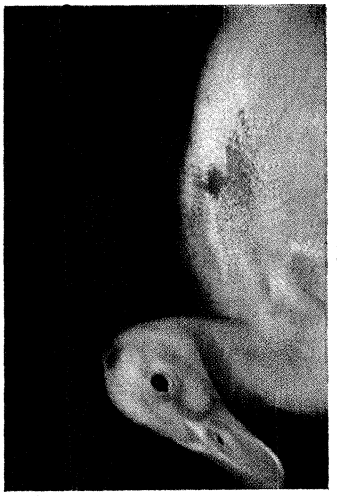

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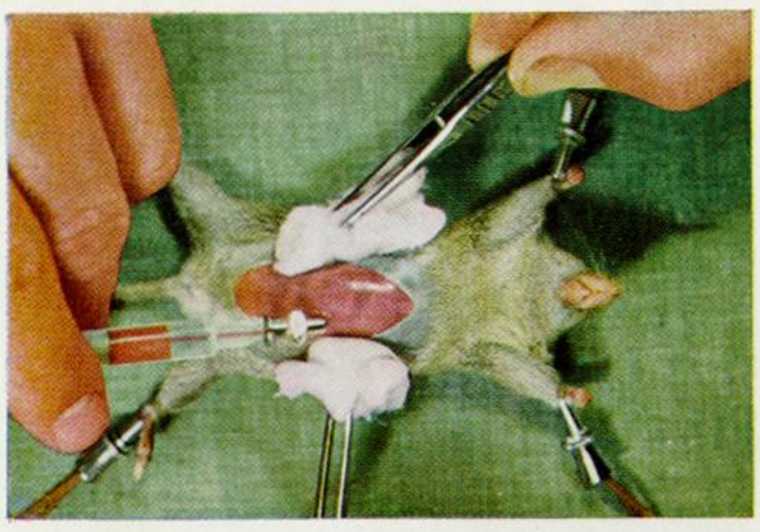

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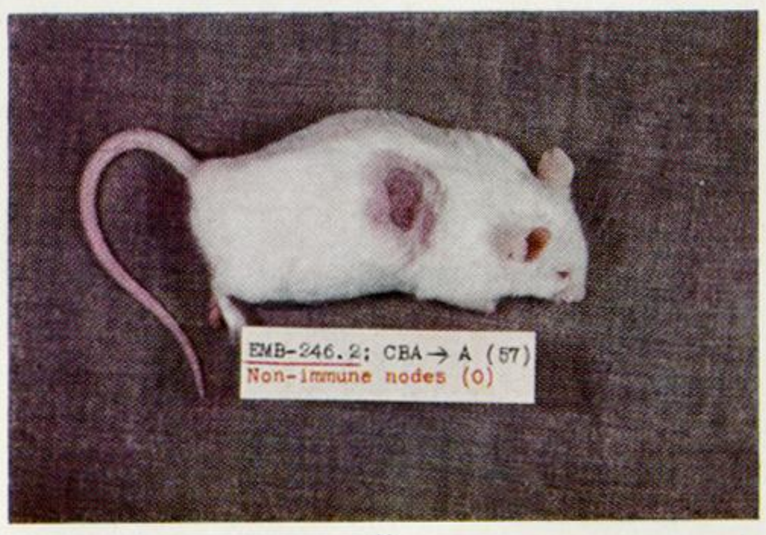

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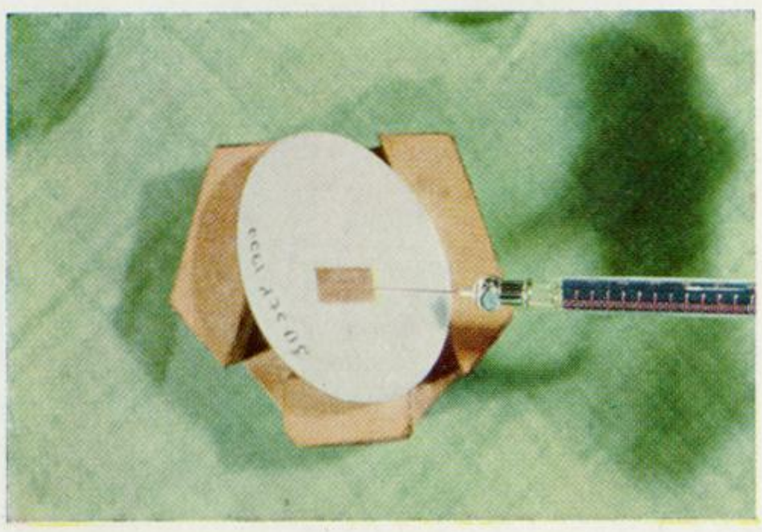

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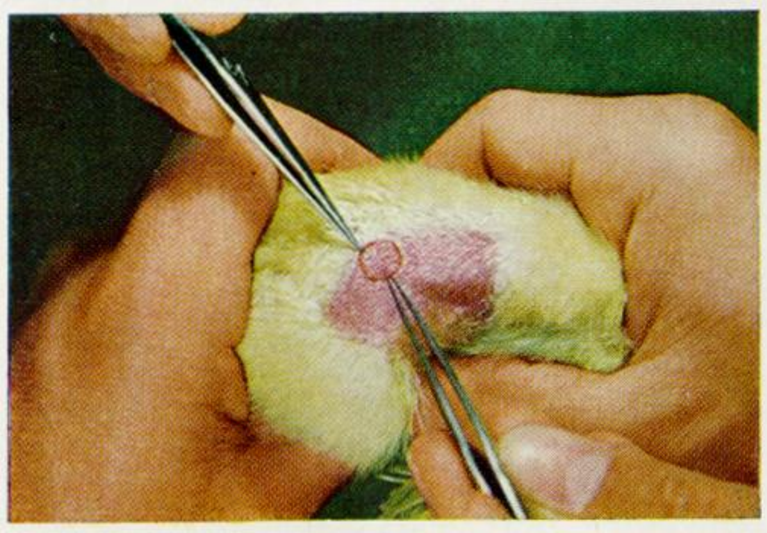

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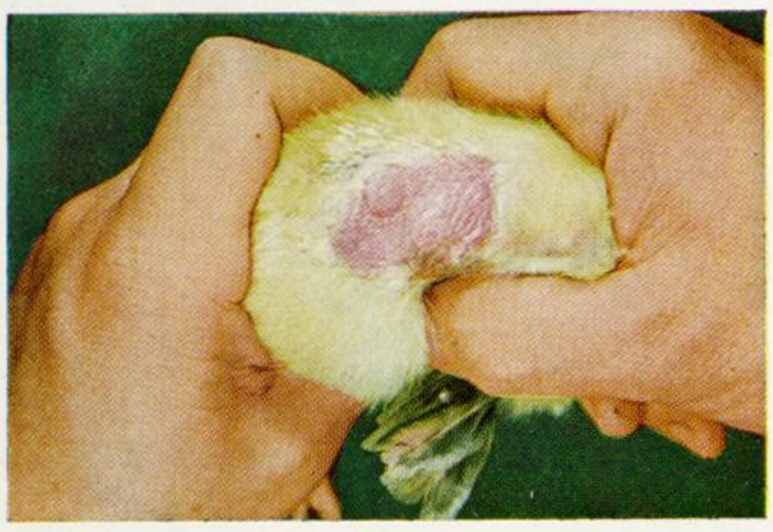

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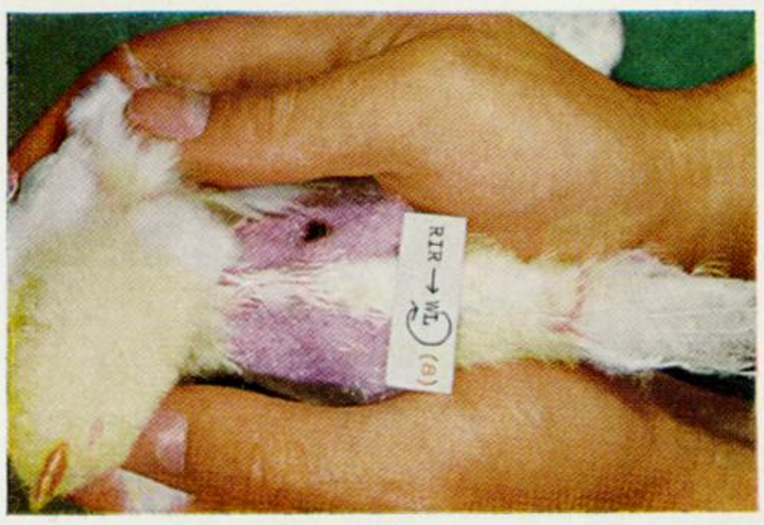

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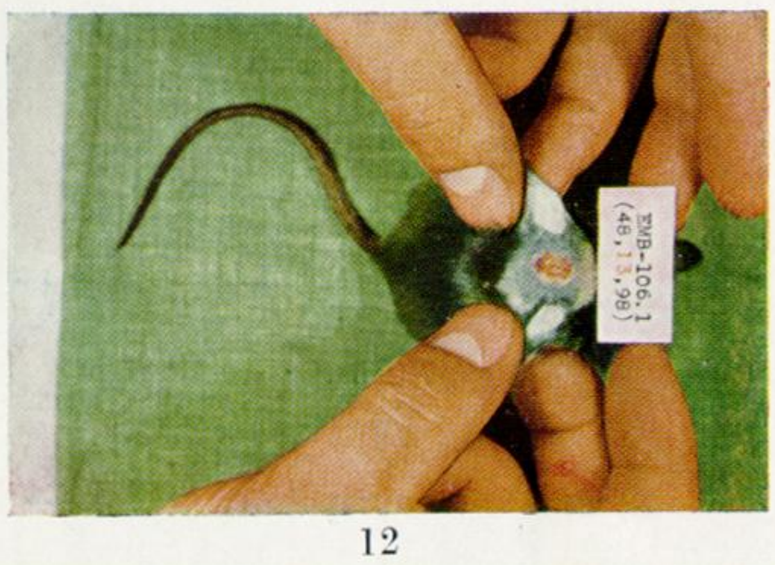

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Plate 7

Figure 1. The technique of injecting mouse embryos through the body wall without laparotomy. The skin has been opened down the ventral midline and gently parted; 17-day embryos have been brought into view and are being injected with a suspension of adult tissues through a fine needle attached to a micrometer-controlled syringe (§3·2.)

Figure 2. The appearance of an *A*-line skin homograft 11 days after transplantation to a normal adult *CBA* mouse. Breakdown is complete, and the graft is drying in air to form a scab. Contrast figures 3 to 6. (§3·2.)

Figure 3. *A*-line skin homografts on a group of tolerant adult *CBA* mice belonging to a single litter, the members of which had been injected *in utero* with an adult *A*-line tissue suspension. Each mouse bears two homografts, that on the right transplanted 83 days beforehand, that on the left 50 days later. The grafts are perfectly normal. Contrast figure 2. (§3·2.)

Figures 4, 5. A *CBA* skin homograft 57 days after transplantation to a tolerant adult *A*-line mouse which had been injected during uterine life with an adult *CBA* tissue suspension. The graft is shown before (figure 4) and after (figure 5) clipping away its pelt of normal agouti hairs: it has been fully incorporated into the skin of its host. (For the later history of this graft, refer to figure 15.) (§3·2.)

Figure 6. An *AU* (black-haired) skin homograft 52 days after transplantation to an *A*-line mouse which had been injected *in utero* with whole blood from an adult *AU* donor. (§3·2.)

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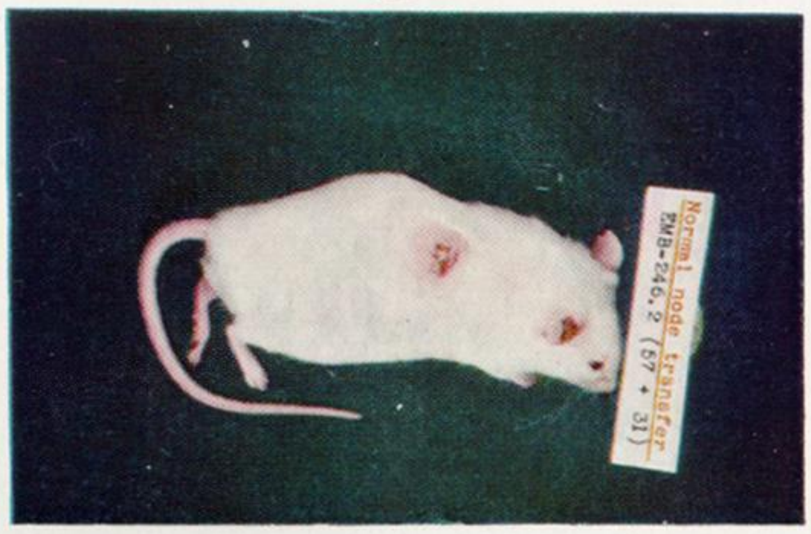

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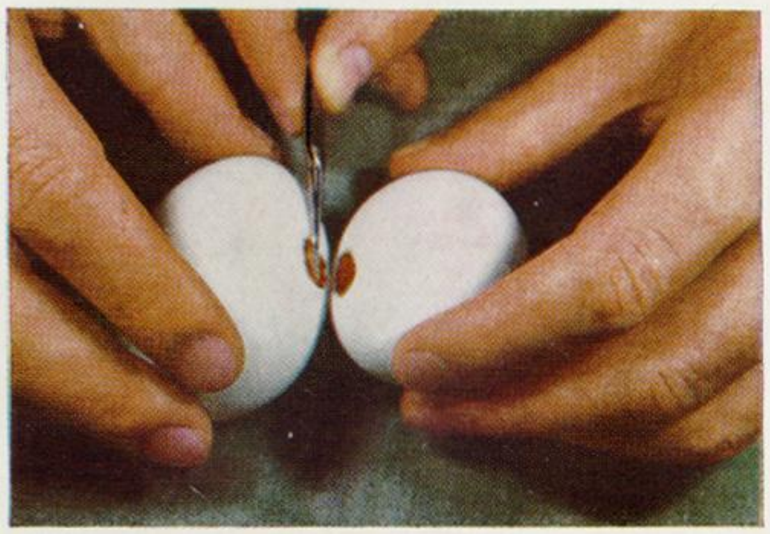

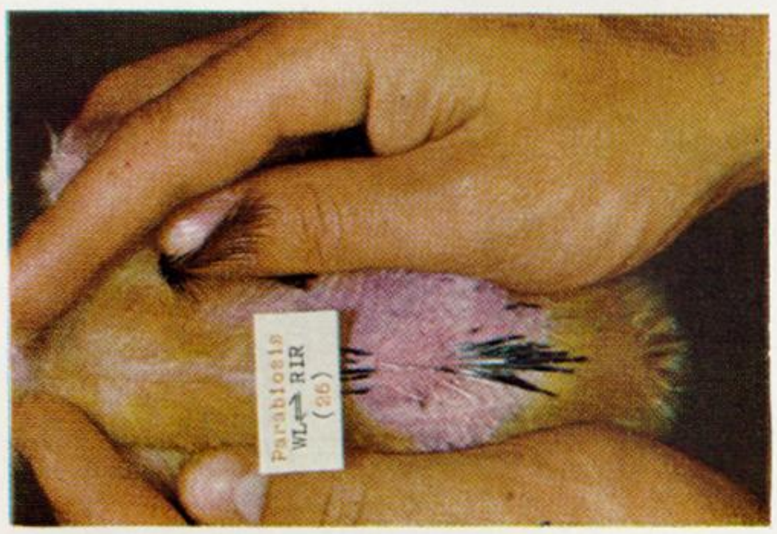

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