In these experiments the regenerative ability of half limbs was also examined (Table 1). In each animal in which one double half limb was produced, the limb from which the graft was taken remained as a half limb, either half anterior or half posterior. These half limbs, as well as half limbs produced on both forelimbs of the same animal were either amputated immediately through the most distal portion of the half limb, or amputated 3–4 weeks later. Most of these limbs developed complete and normal regenerates, thus confirming earlier findings that half limbs can regenerate normally[7,8] or relatively normally[9]. The initial outgrowth did not arise at the most distal cut end of the half limb, but was eccentrically located towards the distal end of the lateral wound edge. During the later stages of regeneration, the regenerate became more symmetrically disposed until by the stage of late digits, little or no asymmetry could be detected. A tentative interpretation of this finding is that the eccentric location of the initial outgrowth is the position on the wound surface at which cells comprising a complete circle of positional values come together during wound healing from the proximal and distal half limb stumps. This possibility is being investigated.

The many similarities between the regulative behaviour of insect and amphibian limbs have led to the hypothesis that the mechanisms of pattern regulation in these distantly related organisms are the same[1]. This idea is strengthened by the similarity of the results reported here to those reported by Bohn[10] with double dorsal and double ventral cockroach legs.

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Detection and repair of single-strand breaks in nuclear DNA

DIRECTLY or indirectly phosphodiester bonds in DNA are broken when living cells are irradiated by ionising radiations or ultraviolet light. There are various sophisticated techniques for monitoring radiation damage[1–6]. We describe here how radiation damage in DNA and its repair can be detected simply in the white cells of human blood. The method is very sensitive and should prove useful in screening populations for abnormal repair mechanisms. As there is also great interest in methods for detecting environmental agents that damage DNA[2,7], we have applied the method to detect the damage caused by mitomycin C.

We have described our technique for detecting the damage caused to the nuclear DNA of HeLa cells by very low doses of γ rays and also for monitoring the repair of this damage[8,9]. When cells are lysed in the presence of non-ionic detergents and high salt concentrations, structures resembling nuclei—called nucleoids—are released. These nucleoids contain nearly all nuclear RNA and DNA, but are depleted of nuclear proteins[10]. Their DNA is supercoiled and compact so that the nucleoids sediment more rapidly in sucrose gradients than their γ-irradiated counterparts which contain extended DNA with single-strand breaks or nicks. Repair of the breaks restores the DNA to its original conformation and re-establishes the normal sedimentation rate. We monitor DNA integrity by measuring the distance sedimented by nucleoids in sucrose gradients.

Nucleoids are released immediately on addition of white blood cells to a lysis mixture containing the non-ionic detergent Triton X-100 and 1.95 M NaCl. The effects of the intercalating dye, ethidium bromide (EB), on the sedimentation of nucleoids derived from white blood cells was studied by spinning the nucleoids through sucrose gradients containing 1.95 M NaCl and different concentrations of the dye (Fig. 1). The distance travelled by the nucleoids is expressed as a ratio relative to that travelled by unirradiated nucleoids sedimenting in the same conditions but in the

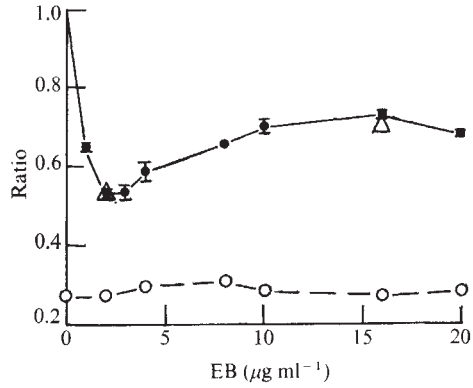

Fig. 1 Effect of EB on the sedimentation of nucleoids from human white blood cells. The distance sedimented by nucleoids in gradients containing different concentrations of EB is expressed as a ratio relative to that of unirradiated nucleoids sedimenting in the absence of EB. ●, Unirradiated nucleoids; ○, nucleoids γ irradiated (960 rad, 120 rad min^{-1}) after addition of cells to the lysis mixture[8]; △, unirradiated nucleoids from a patient with xeroderma pigmentosum. Error bars give the standard error of the mean. Human white cells were obtained from peripheral blood[17] and cultured in RPMI 1640 medium supplemented with 20% foetal calf serum (Flow Laboratories). Between 1 and 5% of the cells prepared in this way are red cells, the remainder are almost all lymphocytes. The results obtained with blood from four normal males and two females were pooled. In some cases white cells stored in medium on ice for 24 h were used; the sedimentation properties of nucleoids prepared from such cells were similar to those obtained from freshly isolated cells. Samples of 50 μl of a suspension containing between 2 and 5×10^5 cells in phosphate-buffered saline (PBS) were layered on 150 μl of a lysis mixture floating on top of "isokinetic" sucrose gradients[8,10]. Gradients (15–30% sucrose, 4.6 ml, *p*H 8.0) contained 1.95 M sodium chloride, 0.01 M Tris, 0.001 M EDTA in addition to various concentrations of EB. The lysis mixture contained sodium chloride, EDTA, Tris and Triton X-100 in amounts which, on addition of 1 volume of PBS containing cells to 3 volumes of the mixture, gave final concentrations of the constituents of 1.95 M, 0.1 M, 2 mM and 0.5% respectively. (For the purpose of calculating the final concentration of sodium chloride, the contribution of the PBS is neglected). Fifteen minutes after the addition of the cells to the lysis mixture, gradients were spun at 30,000 r.p.m. for 25 min at 20 °C in the SW50.1 rotor in a Beckman L2-65b ultracentrifuge. After the gradients had been spun, the position of the nucleoids in the gradient was determined by their absorbance at 254 nm. One gradient of the six spun in the rotor served as a reference; the distance travelled by nucleoids in other tubes is expressed relative to the distance sedimented by nucleoids in the reference tube[8].

absence of EB. As the concentration of EB in the gradient is increased, the distance travelled by nucleoids falls to a minimum and then rises again. γ Irradiation reduces the sedimentation rate of the nucleoids and abolishes the biphasic response to EB. The shape of a similar curve obtained with HeLa nucleoids is discussed elsewhere[8]. As the sedimentation rate of supercoiled DNA varies in this characteristic biphasic manner[11], we conclude that nucleoid DNA is supercoiled. Irradiation introduces single-strand breaks into the DNA so that supercoils are lost.

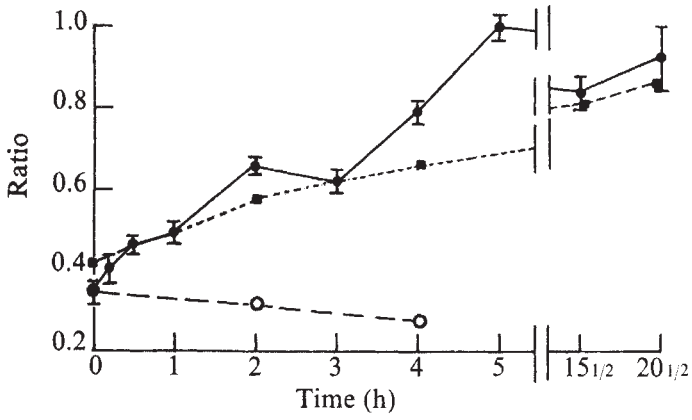

Fig. 2 Effect of incubation of white blood cells after γ irradiation on nucleoid sedimentation. The distance sedimented by nucleoids from irradiated cells in gradients lacking EB is expressed as a ratio relative to that of nucleoids from unirradiated cells which had been treated similarly. White cells (2×10^6 per ml in medium) were irradiated (960 rad)[8], and incubated for different times at 37 or 4 °C. They were then added to 10 volumes of ice-cold PBS, pelleted and resuspended in ice-cold PBS. Samples of 50 μl were applied to gradients which were then spun and analysed as described in the legend to Fig. 1. Error bars give the standard error of the mean. Normal white blood cells, irradiated and incubated at 37 °C (●) or 4 °C (○). White cells from a patient with xeroderma pigmentosum, irradiated and incubated at 37 °C (■).

Repair of the damage caused by γ irradiation was studied by irradiating white blood cells (960 rad) and then incubating them for different periods before applying them to gradients lacking EB and then measuring the sedimentation rate of the nucleoids. (A dose of 960 rad reduces the cloning efficiency of HeLa cells by 99% (ref. 8.) Incubation at 37 °C after irradiation, but not at 4 °C, increased the sedimentation rate of the nucleoids (Fig. 2). After 5 h, the sedimentation rate had been restored almost to that of unirradiated nucleoids, and the nucleoids from the irradiated cells had regained their biphasic response to EB (Table 1).

These techniques have been extended to include measurements on the effects of ultraviolet light. Repair of damage induced by ultraviolet light involves cutting one strand of the DNA duplex ("incision"), removal of the principal photoproduct, the thymine dimer ("excision"), synthesis of DNA complementary to the unaffected strand and "ligation" of the final phosphodiester bond to restore the intact duplex[5]. HeLa cells were irradiated (25 erg mm^{-2}) and incubated for different times at 37 °C before the sedimentation rate of nucleoids isolated from them was measured in gradients lacking EB (Fig. 3*a*). (A dose of 25 erg mm^{-2} reduces the cloning efficiency of HeLa cells by 24% (our unpublished results).) Irradiation without subsequent incubation at 37 °C reduces the rate of sedimentation. This probably results from the single-strand breaks induced by the highly active incision enzymes of HeLa cells during the time taken to concentrate the cells before they are lysed. (Although the formation of a thymine dimer in DNA unwinds the double helix by 5–6° (ref. 12), the sedimentation rate is unlikely to be reduced by such unwinding in the absence of strand scission because irradiation of isolated nucleoids with high doses of ultraviolet light does not alter the sedimentation rate (our unpublished results).) Incubation of the cells at 37 °C for 5 min after irradiation reduced the rate of sedimentation of the nucleoids further, whereas incubation periods greater than 20 min increased the distance sedimented until it became indistinguishable from that of the unirradiated controls. We conclude that after irradiation, incision initially leads to a partial loss of supercoils in nucleoid DNA, but then ligation restores supercoiling (Fig. 3*a*).

A measure of the damage caused by different doses of ultraviolet light is given by the distance sedimented in the absence of EB by nucleoids after the irradiated cells had been incubated for 5 min at 37 °C to maximise the number of strand breaks (Fig. 4). As radiation dose increased, the distance sedimented decreased progressively.

The damage caused by ultraviolet light is repaired much more slowly in white blood cells than in HeLa cells. Strand breakage and loss of supercoiling were maximal 2 h after irradiation. The normal supercoiled configuration was not fully recovered after 15.5 h (Fig. 3*b* and Table 1). There was

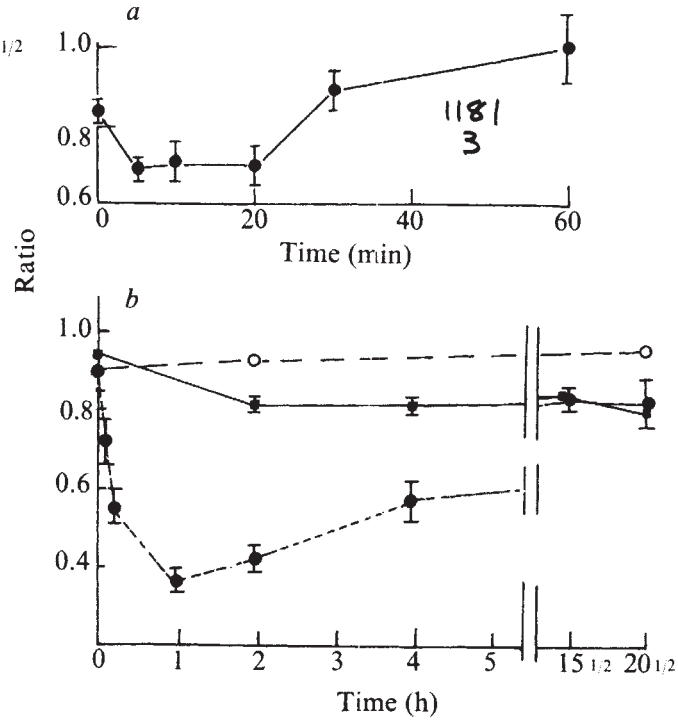

Fig. 3 Effect on nucleoid sedimentation of incubating (*a*) HeLa cells and (*b*) human white blood cells at 37 °C after irradiation with ultraviolet light. *a*, Human HeLa cells were grown in suspension[8]. 0.5 ml of cells (2×10^6 per ml) in ice-cold medium in a Petri dish were irradiated (25 erg mm^{-2}, 5 erg mm^{-2} s^{-1}) with ultraviolet light while the Petri dish was shaken vigorously. The source of ultraviolet light was a Sylvania germicidal tube (G 15T8). Doses were measured with an ultraviolet meter[18]. After irradiation, cells were mixed with 4.5 ml of medium at 37 °C and incubated in the dark at 37 °C. At the end of incubation the cells were mixed with 9 volumes of ice-cold PBS, pelleted and resuspended in ice-cold PBS. Samples of 50 μl of the cell suspension were applied to sucrose gradients lacking EB. Gradients were spun at 5,000 r.p.m. for 50 min and analysed as described in the legend to Fig. 1. The distance sedimented by nucleoids derived from irradiated cells is expressed as a ratio relative to the distance sedimented by nucleoids derived from cells treated similarly but which had not been irradiated. Error bars give the standard error of the mean. *b*, The experiments with white blood cells were similar to those with HeLa cells except that media and spinning conditions were different (see legend to Fig. 1). ●, Normal white blood cells, irradiated and incubated at 37 °C; ○, normal white blood cells, irradiated and incubated at 4 °C; ■, white blood cells from a patient with xeroderma pigmentosum, irradiated and incubated at 37 °C.

Table 1 Effect of different treatments on supercoiling in the nucleoids from white blood cells

Treatment	Relative distance sedimented by nucleoids in the presence of EB			
	0 μg ml^{-1}	2 μg ml^{-1}	4 μg ml^{-1}	16 μg ml^{-1}
Untreated	1.0	0.53	0.59	0.73
4-h incubation at 37 °C after γ irradiation (960 rad)	0.82	0.53	0.66	0.65
2-h incubation at 37 °C after ultraviolet irradiation (25 erg mm^{-2})	0.39	0.39	—	0.35
15.5-h incubation at 37 °C after ultraviolet irradiation (25 erg mm^{-2})	0.84	0.58	—	0.68

Nucleoids obtained from cells treated in different ways were spun in gradients containing different concentrations of EB. The distance sedimented by nucleoids from treated cells is expressed as a ratio relative to that of nucleoids from untreated cells sedimenting in the absence of ethidium. The conditions used for the untreated cells, and cells irradiated with γ rays or ultraviolet light are described in the legends to Figs 1, 2 and 3, respectively.

little change in the sedimentation rate of nucleoids when the irradiated cells were incubated at 4 °C (Fig. 3*b*).

Cells from patients with the autosomal recessive disease, xeroderma pigmentosum can repair the damage caused by ionising radiation but cannot effectively repair DNA damaged by ultraviolet light. Some forms of this disease are thought to be characterised by deficiency in "incision" activity[5]. Nucleoids prepared from the white cells of our patient sedimented in gradients containing EB exactly as the nucleoids from normal cells (Fig. 1). In the absence of EB the sedimentation behaviour of nucleoids prepared from γ-irradiated cells was also indistinguishable from that of nucleoids prepared from normal cells that had been irradiated (Fig. 2): the patient's cells could thus repair γ-ray damage. On the other hand, irradiation with ultraviolet light affected normal cells and those of the patient differently (Fig. 3*b*). When the patient's cells were irradiated with ultraviolet light and incubated at 37 °C the sedimentation rate of the nucleoids in the absence of EB was not reduced as much as that of controls : the patient's cells were unable to "incise" normally and the nucleoids retained supercoils and sedimented rapidly.

Mitomycin C is a carcinogen[7]. After activation by cellular enzymes it cross links DNA strands without breaking them. Strands are broken only when the damaged region is removed by repair enzymes[13]. We investigated the effects

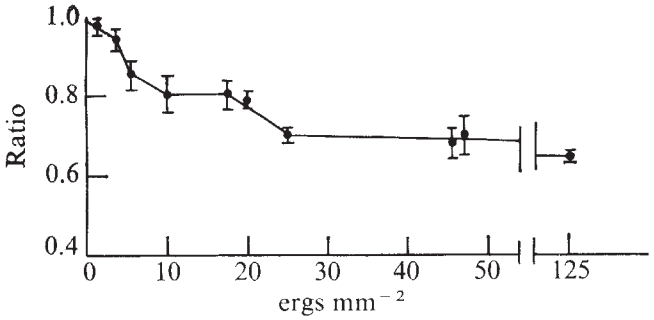

Fig. 4 Effect of different doses of ultraviolet radiation on the sedimentation of HeLa nucleoids. HeLa cells were irradiated for 5 s with different doses of ultraviolet light, incubated for 5 min at 37 °C, applied to sucrose gradients lacking EB and the gradients were spun and analysed (see legend to Fig. 3). The distance sedimented by nucleoids derived from irradiated cells is expressed as a ratio relative to the distance sedimented by nucleoids derived from cells which had been treated similarly but which had not been irradiated. Error bars, s.e.m.

of mitomycin C on the integrity of DNA by incubating white cells at 37 °C for 2 h in different concentrations of the drug, lysing the cells, and determining the rate of sedimentation of the nucleoids in the absence of EB (Fig. 5). Low concentrations of the drug had no effect on the distance sedimented, higher concentrations reduced it. At the high concentrations, strand breakage caused loss of supercoiling and slow sedimentation. The effects of lower concentrations can be detected by incubating the cells with the drug for longer periods (our unpublished results). If, after a 2-h exposure to the drug, the cells were washed and incubated in its absence at 37 °C for 15.5 h, faster sedimentation was restored, repair of the damage restored normal supercoiling.

We have made nucleoids containing superhelical DNA from a wide range of cells (fibroblasts, lymphoblasts, erythroblasts, teratocarcinoma and epithelial cells of men, mice, birds, amphibians and insects (ref. 9 and our unpublished observations)), so that these methods for detecting single-strand breaks in DNA should be applicable to most cells.

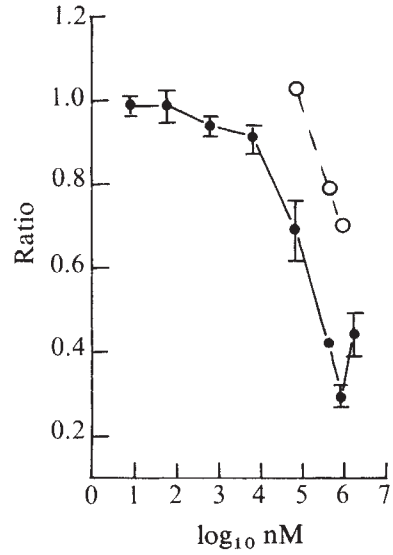

Fig. 5 Effect of mitomycin C treatment of white cells on the sedimentation of nucleoids in gradients lacking EB. White cells were incubated for 2 h at 37 °C (see legend to Fig. 1) in the presence of different concentrations of mitomycin C (Boehringer). Some cells were then collected and applied to gradients immediately and the gradients were spun and analysed (see legend to Fig. 1). Other cells were washed twice with medium, resuspended at 2×10^6 per ml in medium lacking mitomycin C and incubated a further 15.5 h at 37 °C. They were then collected and treated as the others.. The distance sedimented by nucleoids derived from cells treated with mitomycin C is expressed as a ratio relative to the distance sedimented by those derived from untreated cells. Error bars, s.e.m. ●, Cells incubated for 2 h in the presence of mitomycin C. ○, Cells incubated for 2 h in the presence of mitomycin C, washed and then incubated for a further 15.5 h at 37 °C.

Using white cells obtained from human blood taken in the morning, we can assess by the evening whether repair of damaged DNA in the cells is normal. While doses of 960 rad (γ rays) or 25 erg mm^{-2} (ultraviolet light) produce the large effects described in these experiments, much lower doses (that is, 12 rad and 5 erg mm^{-2}) can be detected. The method should therefore be particularly well suited to the rapid screening of patients for deficiencies in the mechanisms that repair radiation damage.

Many carcinogens and mutagens break phosphodiester bonds in cellular DNA either directly or indirectly after enzyme action. Breakage, which can be demonstrated by

conventional physical techniques[14] probably increases repair synthesis[15] and recombination[2,16]. Agents that break DNA can be detected in nucleoids with the sensitivity, rapidity and economy usually associated with the use of bacteria[7]. There may well be advantages in using human cells in any screening method for detecting potential human carcinogens.

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Evidence of a thymic abnormality in murine muscular dystrophy

The involvement of the thymus in muscular, neuromuscular and neurological disorders is well documented[1–4]. In spite of the extensive study made of the thymus in myasthenia gravis, which, as a neuromuscular disorder with clinical symptoms of muscular weakness, shares some similarities with muscular dystrophy, the state and function of the thymus in muscular dystrophy has been ignored. The poor health and susceptibility to infection of dystrophic mice have frequently been observed. These observations and the similarities between dystrophic wasting and that seen in mice thymectomised pre- or neonatally[5], led us to the conclusion that an investigation into the status of the thymus in murine muscular dystrophy was warranted. We have found that the thymuses of dystrophic mice exhibit changes in the age-dependent variation in thymic weight, together with aberrations in cellular morphology indicative of altered secretory activity.

Male and female dystrophic mice of the Bar Harbor 129/ReJ strain were killed by cervical dislocation at various ages, their thymuses, spleens and elbow lymph nodes removed, dissected free of fat and connective tissue, weighed and fixed in 10% formol-saline or methanol. The thymuses, spleens and lymph nodes from clinically normal littermates, and also from a non-dystrophic strain of mice (CBA), were used for comparison. The organs were paraffin embedded and subjected to routine haematoxylin–eosin staining procedures. Unna–Pappenheim staining was also used[6]. The histology of random single sections of these organs was then compared.

For an initial indication of thymic status, the thymus weights of normal and dystrophic mice were compared. It is not possible to compare thymic indices in these animals because of the selective wasting of hind limb muscle in the dystrophic mouse. Generally, mouse thymuses have been found to decline in weight from 4 to 12 weeks of age; the thymuses of the normal littermates followed this trend. In dystrophic mice, the thymic weights were at first much lower, but then increased with age, reaching a maximum at approximately 9 weeks of age, at a weight comparable to that of the normal mouse at 4–6 weeks of age. The thymic weights of the dystrophic mice then slowly declined to a level approaching that of their normal littermates at 12 weeks of age (Fig. 1). In all strains of mice so far studied, there has been to our knowledge, no report of a similar weight gain to 9 weeks of age, the predominant pattern being an increase in weight to 4 weeks of age,

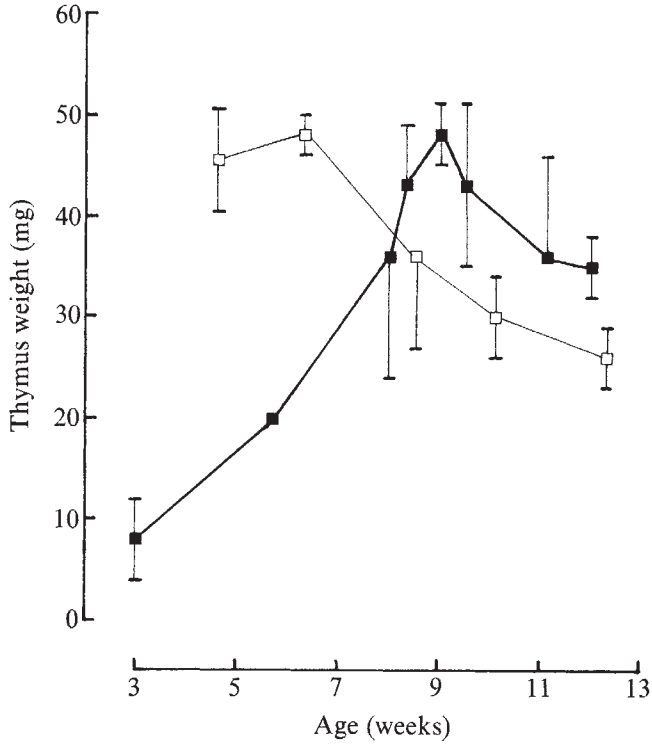

Fig. 1 Thymus weight as a function of age in normal and dystrophic mice. Thymus weight (mg)±s.d. Normal mice, 4–7 animals per group. Dystrophic mice, 2–6 animals per group.

followed by a gradual, but definite, decrease in weight which continues throughout the life span of the animal[7].

This anomaly led us to consider the histology of the thymus of dystrophic mice. As shown in Fig. 2, this also differed dramatically from the normal. While the normal littermates showed no abnormalities of basic thymic morphology, a number of the dystrophic animals studied exhibited remarkable alterations of thymic cellular composition. In the main, this consisted of a depletion, and in some cases, total absence of lymphocytes from lobules of the thymus, these being replaced by epithelial cells which showed very marked signs of secretory activity[8]. In extreme cases, the epithelial cells had formed themselves into classical, though duct-less acini, surrounding a material which did not stain in the conditions used. These drastic changes in thymic composition were found in young (3–8 weeks of age) dystrophic mice which were severely affected by the disease, as evidenced by the degree of disability. In older animals, and those less severely affected, the thymus showed less dramatic changes. These consisted of an apparent relative increase in medullary area over cortical area and a loss of distinction of the cortico-medullary junction. The change in the ratio of medulla to cortex could be due to an increase in medullary tissue or a decrease in the number of lymphocytes populating the