*J. Physiol.* (1973), **229**, *pp.* 681–695
*With 6 text-figures*
*Printed in Great Britain*



# THE INFLUENCE OF EXOGENOUS STEROIDS ON MACROMOLECULE UPTAKE BY THE SMALL INTESTINE OF THE NEW-BORN RAT

By V. G. DANIELS,* R. N. HARDY, K. W. MALINOWSKA, AND P. W. NATHANIELSZ

*From the Physiological Laboratory, Downing Street, Cambridge CB2 3EG*

(*Received 17 August 1972*)

SUMMARY

1. Plasma concentrations of cortisol and corticosterone measured by competitive protein binding in rats between 5 and 28 days after birth have been related to the intestinal uptake of [$^{125}$I]polyvinyl pyrrolidone (PVP).

2. Plasma cortisol concentration was consistently low throughout the period studied, but there was an increase in plasma corticosterone concentration at the time (18–21 days) when PVP uptake declined to zero (closure).

3. Injection of a large dose of cortisone acetate 5 days after birth resulted in precocious closure; PVP uptake declined progressively to zero during the 6 days following the injection. Injection of this steroid at 12 days of age caused closure within 4 days.

4. Precocious closure induced by cortisone acetate was closely comparable histologically with natural closure; the decline in PVP uptake was associated with the progressive displacement of vacuolated cells from the villi of the terminal intestine.

5. Injection of corticosterone at either 5 or 12 days after birth also reduced PVP uptake. However, the reduction was transient and uptake returned to control levels some days after the injection.

6. The temporary reduction in PVP uptake following corticosterone injection was not associated with any change in the histological appearance of the small intestine at the light microscope level.

7. The injection of either cortisone acetate or corticosterone was followed by a period of impaired body growth and also a reduction of adrenal weight in animals injected at 12 days but not in animals injected at 5 days.

* Present address: Department of Biology, Open University, Bletchley, Bucks.

## INTRODUCTION

During the first 18 days after birth the small intestine of the suckling rat can take up maternal antibodies from the colostrum and milk and transfer them into the circulation, but between 18 and 21 days after birth this transfer decreases dramatically to zero ('closure') (Halliday, 1955).

Closure probably represents the failure of the antibodies to enter the villous epithelial cells since the intestinal uptake of the inert macromolecule, [$^{125}$I]polyvinyl pyrrolidone (PVP) declines concurrently with failure of antibody transfer (Clarke & Hardy, 1969*a*). The decrease in PVP uptake between 18 and 21 days after birth has been shown to be the result of the progressive replacement of the vacuolated villous epithelial cells capable of taking up PVP by more mature non-vacuolated cells which do not have this ability. The duration of the declining phase of PVP uptake thus corresponds with the time necessary completely to replace the villous epithelium (about 3 days) (Clarke & Hardy, 1969*b*).

Little is known of the normal physiological stimulus to the crypts of Lieberkühn which results in the production of the more mature type of epithelial cell, but three lines of evidence have accumulated to indicate, in the rat at least, that the adrenal cortex may be implicated. First, the observation that closure can be induced up to 9 days prematurely by the administration of large doses of cortisone acetate or deoxycorticosterone acetate given either parenterally or orally (Halliday, 1959; Clark, 1959); secondly, the delay in closure which results from bilateral adrenalectomy (Daniels & Hardy, 1971, 1972; Daniels, Hardy & Malinowska, 1973); and, thirdly, the increase in plasma corticosterone concentration which normally occurs at the time of closure (Daniels, Hardy, Malinowska & Nathanielsz, 1972).

The present investigation represents a re-examination and extension of the original work of Halliday (1959) in an attempt to define more precisely the changes which are associated with the precocious closure induced by the administration of exogenous steroids. In particular, the effects of corticosterone have been examined, since this is the main adrenal steroid in the rat and it has not been previously studied in this context.

## METHODS

### *Animals*

Litters from a closed colony of Carnworth SPF rats were used. They were reared in a quiet animal-house room, maintained at 20° C and with a single period of 14 hr light in each 24 hr. Mothers received pellets of diet 41B (Oxoid) *ad lib* and tap water *ad lib* to which had been added chlordiazepoxide hydrochloride (Librium, Roche), 20 mg/l. to reduce cannibalism (Helyer & Howie, 1963).

The working day on which the litters were first noted was called day 1; thus for litters born after 5.30 p.m. the following day was day 1.

*Feeding procedure*

Young rats were starved for 2 hr to ensure partial emptying of the stomach and were then given, by a fine polyethylene stomach tube, 0·25 ml. of the test solution. This comprised a 2% aqueous solution of PVP K. 60 (Fluka AG) containing 0·25–0·50 $\mu$c [$^{125}$I]PVP K. 60 (Radiochemical Centre, Amersham) and Blue Dextran 2 mg/ml. (Pharmacia). The rats were then returned to the mother and allowed to suckle for 4 hr.

*Measurement of PVP uptake*

Rats were killed by decapitation and a blood sample was taken immediately from the severed neck into a heparinized tube for steroid estimation. The stomach and contents, large intestine and contents and lungs were removed and placed in counting tubes. Assessment of the radioactivity in the lungs provided a check of the feeding procedure, since there was no radioactivity in the lungs of animals in which the stomach tube was correctly positioned and in which no regurgitation had occurred. The radioactive content of the large intestine gave an indication of the transit time of the intestinal contents as discussed by Clarke & Hardy (1969*a*). The small intestine was dissected free of mesentery and flushed through with 0·9% saline to remove free [$^{125}$I]PVP from the lumen, as described previously (Clarke & Hardy, 1969*a*). The amount of [$^{125}$I]PVP remaining in the wall of the small intestine was expressed as a percentage of the amount fed less the amount recovered from the stomach, i.e. as a percentage of the [$^{125}$I]PVP presumed to have entered the small intestine. This quantity will be referred to as the 'percentage PVP uptake' (Clarke & Hardy, 1969*a*).

*Organ weights*

Both adrenal glands were removed immediately after decapitation and weighed to within 0·5 mg.

*Measurement of radioactivity*

Samples were analysed in a Nuclear-Chicago Model 4222 Auto Gamma counting system to an accuracy of 1% or better.

*The measurement of plasma corticosteroid concentrations*

Cortisol and corticosterone in plasma were separated and measured as described previously (Malinowska *et al.* 1972). The samples from cortisone acetate injected animals were further analysed by separating cortisone acetate, cortisone and cortisol on a 60 cm long column (1 cm diameter) of Sephadex LH-20, packed in dichloromethane:methanol (98:2 (v/v)) and eluted with the same solvent (Murphy, 1971).

Cortisone acetate was eluted in the 35–55 ml. fraction, cortisone in the 55–75 ml. fraction, and cortisol in the 75–100 ml. fraction. The eluted fractions were evaporated and measured by the competitive protein binding assay (Murphy, 1967).

## RESULTS

### *Steroid levels during normal closure*

Fig. 1*B* shows the plasma concentration of corticosterone and cortisol from 5 to 28 days after birth. It can be seen that plasma cortisol concentration remained low throughout the neonatal period and did not exceed 1 $\mu$g/100 ml. at any time. The corticosterone concentration, however, first showed a tendency to increase after 16 days, and between 18 and 19 days increased approximately twofold. The mean value then remained between 5 and 7 $\mu$g/100 ml. until day 22, after which time there was a further increase in concentration up to a mean value of about 15 $\mu$g/100 ml. on day 28; this compares with adult male values of $6{\cdot}4 \pm 0{\cdot}5$ $\mu$g/100 ml. (four animals).

The uptake of PVP by the small intestine during the first 28 days after birth is shown in Fig. 1*A*, from which it can be seen that the major decline in PVP uptake, between 18 and 20 days, coincided with the early phase of increase in peripheral plasma corticosterone.

### *Injection of cortisone acetate*

*Effect on PVP uptake.* Fig. 2 shows the PVP uptake measured in groups of rats killed at intervals during the 6 days following a single injection of 5 mg cortisone acetate given 5 days after birth (*A*) and during the 8 days following such an injection given 12 days after birth (*B*).

In animals injected 5 days after birth, PVP uptake fell within 24 hr to less than two thirds of the control value and subsequently continued to decrease until the intestine had completely closed by 6 days after the injection. In animals injected 12 days after birth the decline in PVP uptake relative to controls was even more rapid, so that virtually no PVP was taken up by the intestine 4 days after the steroid injection.

*Effect on weight of the body and adrenal glands.* The injection of cortisone acetate in both 5-day and 12-day-old animals was followed by a period of impaired body growth in comparison with that of control animals (Fig. 3*C*). In animals injected at 5 days the impairment was more marked and more prolonged than in animals injected at 12 days. Thus the former animals 6 days after injection were only approximately half the control weight, whereas at this time after the steroid injection body growth in the 12-day-old animals had recovered and they had attained weights similar to the controls.

Fig. 3 also illustrates the effect of cortisone acetate injection on adrenal weight. In 5-day injected animals, the steroid produced no significant change in absolute adrenal weight compared with controls, whereas in 12-day injected animals there was a significant reduction in adrenal weight

after 48 hr (Fig. 3*A*). The increase in relative adrenal weight in the younger group of rats (Fig. 3*B*) was a consequence of an impairment of whole body growth associated with little effect on the adrenal growth curve. In the 12-day injected rats the reduction in body growth was proportionally less

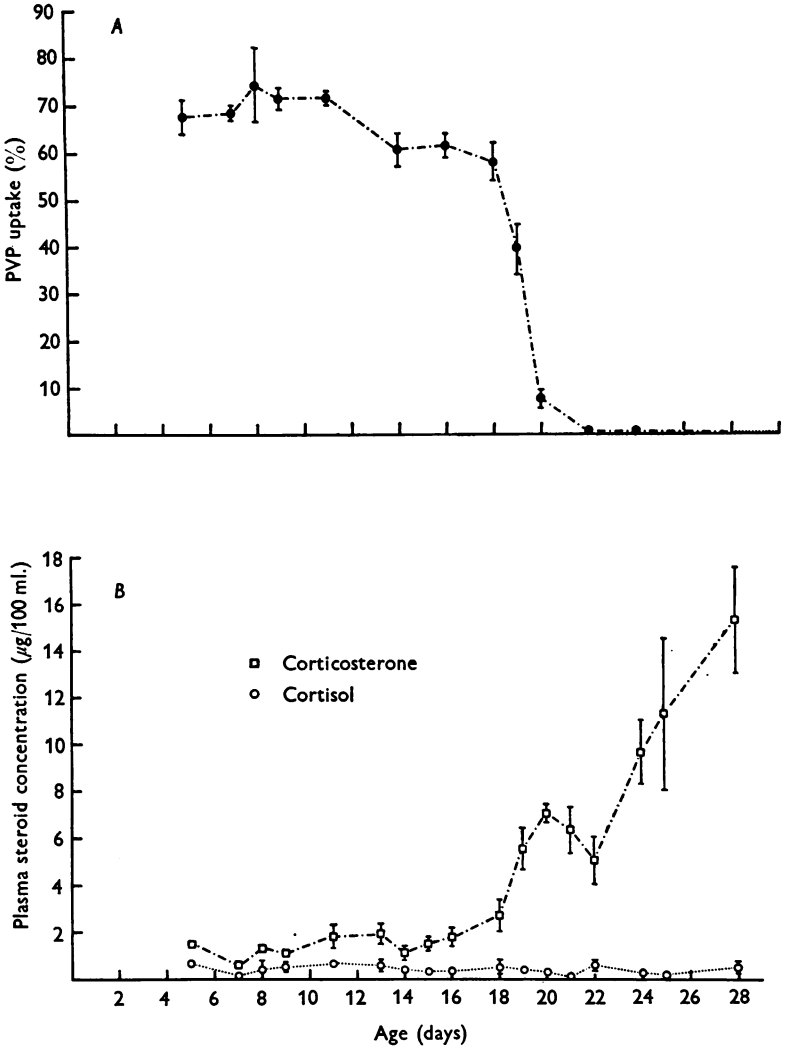


Fig. 1. Relation between intestinal PVP uptake (*A*) and plasma corticosteroid concentrations (*B*) in young rats. Experimental points represent mean values for at least four rats. Vertical lines indicate ± S.E. of mean where this exceeds the dimensions of the plotted point. Ordinates: *A*, % PVP uptake; *B*, plasma steroid concentration μg/100 ml. Abscissae: age after birth in days.

than the atrophic effect on the adrenal so that the relative adrenal weight decreased.

*Injection of corticosterone*

*Effect on PVP uptake.* The time course of the effect on PVP uptake of corticosterone injections at 5 days (2·5 mg) or at 12 days (5 mg) is shown in Fig. 4. In 5-day injected rats the reduction in uptake 24 hr later was comparable with that produced by cortisone acetate at that age (cf. Fig. 2).

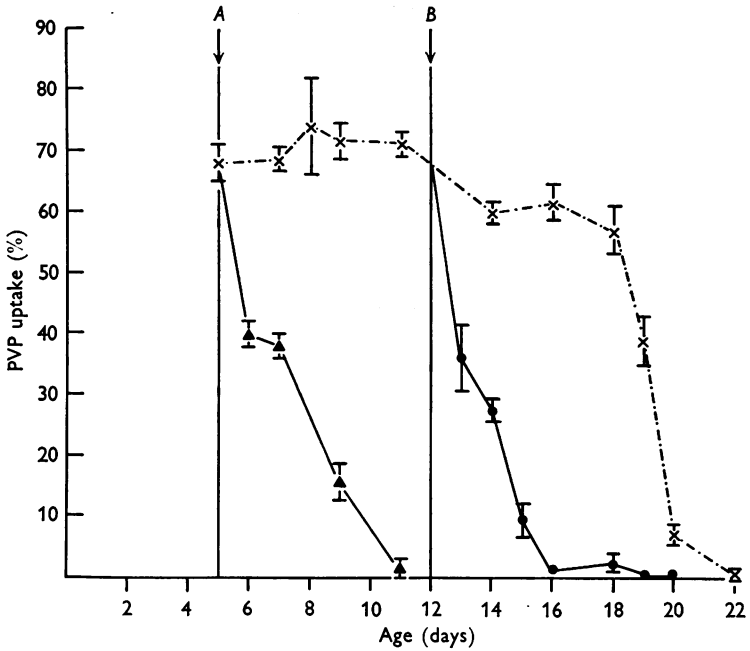


Fig. 2. The effect of cortisone acetate administration on intestinal uptake of PVP. Control PVP uptake ×–·–×; uptake after steroid injection (5 mg) at 5 days after birth, ▲—▲ or 12 days after birth, ●—●. Experimental points represent mean values for at least four rats. Vertical lines represent S.E. of mean where this exceeds the dimensions of the plotted point. Ordinate: percent PVP uptake: abscissa: age after birth (days).

However, uptake on subsequent days was progressively less reduced so that by 6 days after injection (11 days after birth) uptake had returned to control values. In 12-day injected animals the reduction in PVP uptake was also not as profound as that seen in cortisone acetate treated rats, although it was more severe than that produced by corticosterone injection at 5 days; the maximum reduction of uptake was seen 3 days after injection and it took 7–8 days to coincide with control levels.

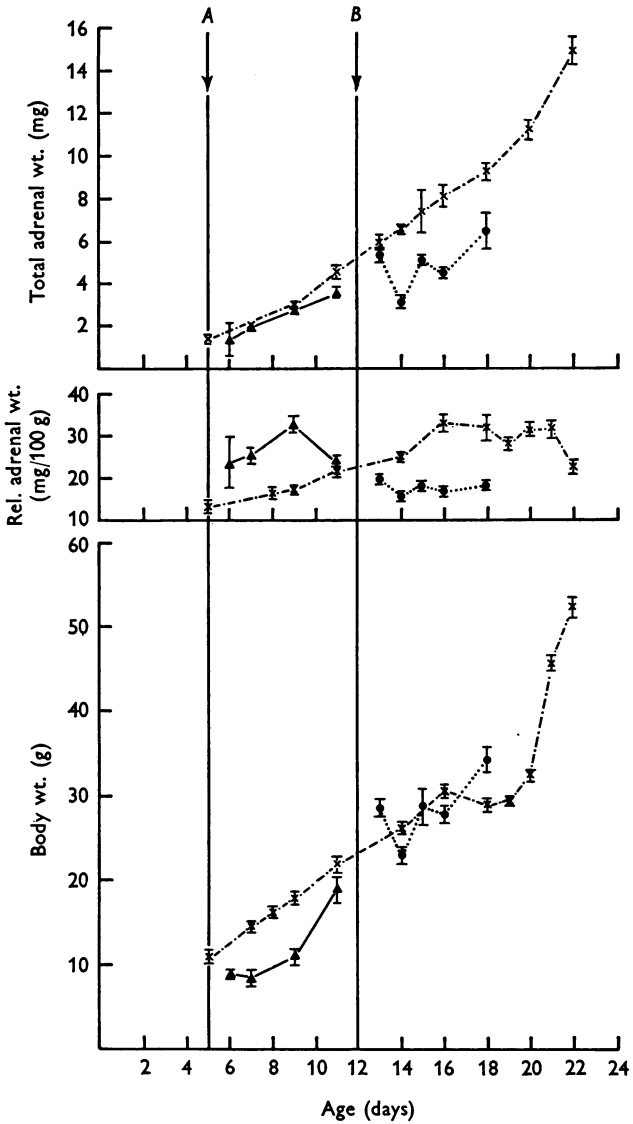


Fig. 3. Effect of cortisone acetate injection at 5 days or 12 days after birth on body weight, total adrenal weight and total adrenal weight relative to body weight. In each panel, control ×---×; injection at 5 days ▲—▲; injection at 12 days ●...●. Experimental points represent mean values for at least four rats. Vertical lines indicate S.E. of mean where this exceeds the dimensions of the plotted point. Ordinates: *A*, weight of both adrenal glands, mg; *B*, relative weight of both adrenal glands, mg/100 g body weight; *C*, body weight (g). Abscissae: age after birth (days).

*Effect on body weight and adrenal growth.* The impairment of growth produced by the injection of corticosterone at 5 days (Fig. 5*C*) was generally comparable with that seen when cortisone acetate was administered (cf. Fig. 3*C*) although the effect was not so prolonged. Injection of corticosterone at 12 days did not, however, produce the reduction in growth seen previously when cortisone acetate was given at this age.

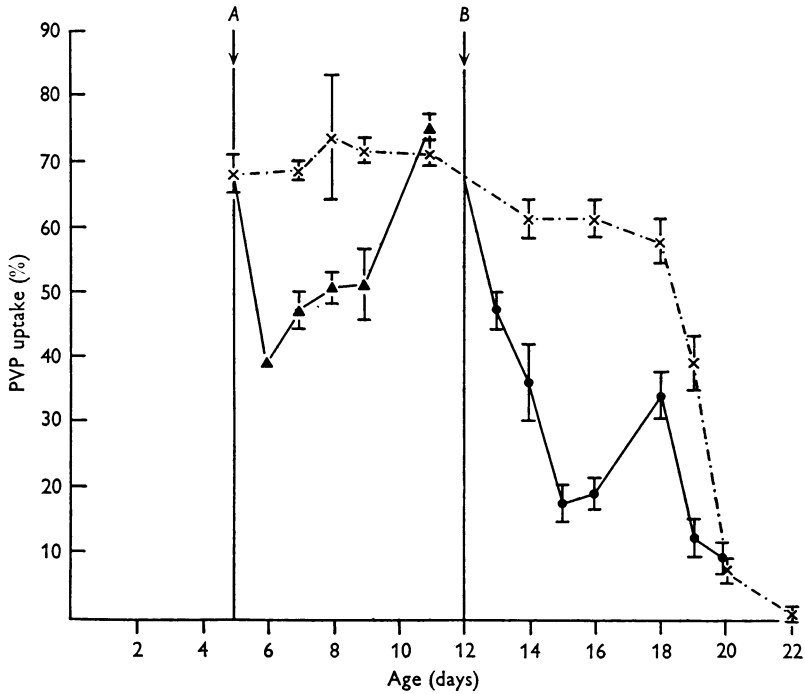


Fig. 4. The effect of corticosterone administration on intestinal uptake of PVP. Control PVP uptake ×···×; uptake after steroid injection, 2·5 mg 5 days after birth, ▲—▲, or 5 mg 12 days after birth ●—●. Annotation as in Fig. 2.

The changes in both absolute adrenal weight and adrenal weight relative to body weight after corticosterone injection at 5 or 12 days were similar to those described after cortisone acetate injection (cf. Fig. 2*A* and *B* with Fig. 5*A* and *B*).

*Plasma steroid concentrations following injection of cortisone acetate or corticosterone*

Steroid concentrations obtained in the period 2–10 hr after S.C. injection of either steroid were high but varied widely from animal to animal. The cause of these variations was almost certainly differences in the rate of

absorption from the injection site. For this reason Fig. 6 shows the mean ± S.E. of mean for all samples obtained during this period.

The plasma concentrations of corticosterone following the injection of corticosterone at either 5 or 12 days after birth are shown in Fig. 6. In

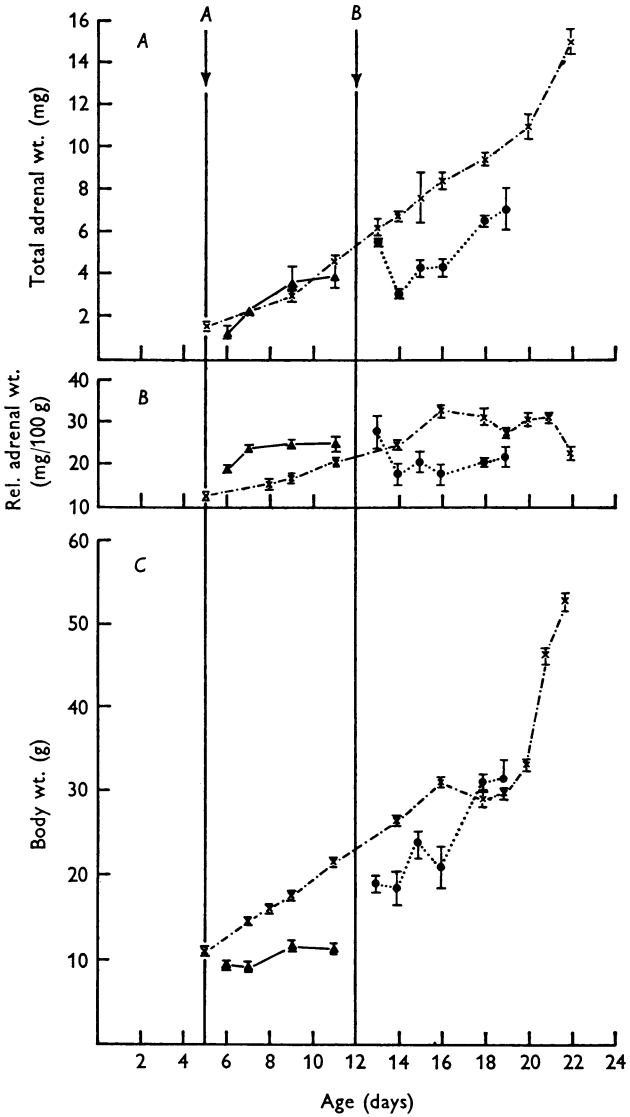


Fig. 5. Effect of corticosterone injection at 5 days (2·5 mg) or 12 days (5 mg) after birth on body weight, total adrenal weight and total adrenal weight relative to body weight. In each panel, controls ×-·-× ; injection at 5 days ▲—▲, injection at 12 days ●....●. Annotation as in Fig. 3.

5-day-old animals injected s.c. with 2·5 mg corticosterone, the mean plasma corticosterone concentration in nine animals killed between 2 and 10 hr after the injection was 123·4 ± 36·7 $\mu$g/100 ml., approximately one hundred-fold above the control values at this age. However, the exogenous steroid was rapidly eliminated, since by 9 days of age corticosterone concentrations were comparable with control levels. In animals injected at 12 days (5 mg) initial plasma levels 2–10 hr after injection were again extremely high (391·4 ± 28·1 $\mu$g/100 ml.) but the concentration fell sharply and control levels were attained within 4 days.

The results of injecting cortisone acetate are also shown in Fig. 6. The

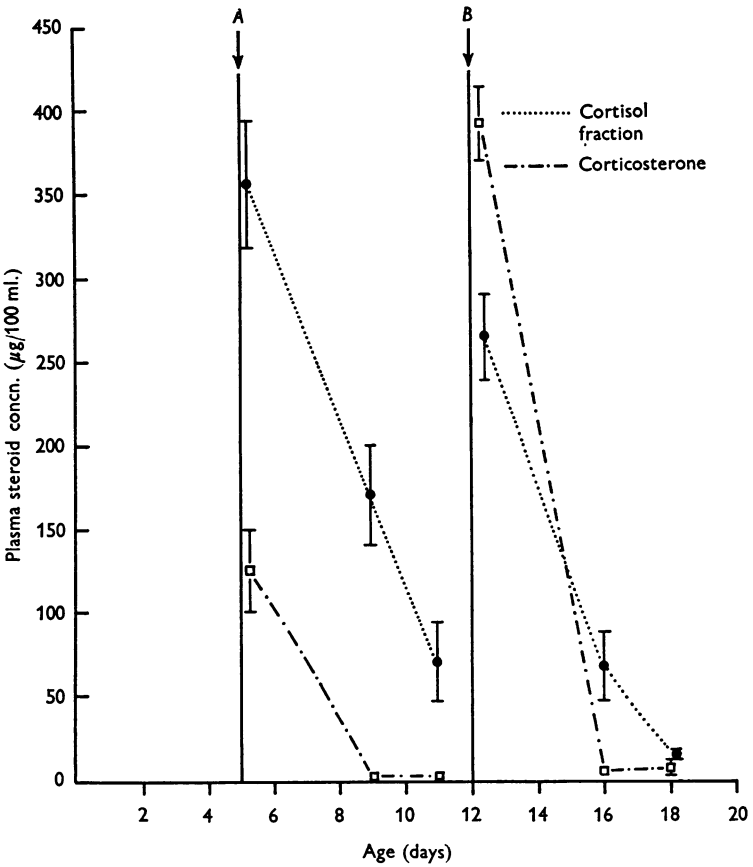


Fig. 6. Plasma concentration of corticosterone and 'cortisol fraction' (see text) following injection of corticosterone or cortisone acetate respectively at 5 days or 12 days after birth. Experimental points represent mean values for at least four rats. Vertical lines indicate ± S.E. of mean where this exceeds the dimensions of the plotted point. Ordinate: plasma steroid concentration $\mu$g/100 ml.; abscissa: age after birth (days).

plasma steroid concentrations in this instance represent the sum total of steroids eluted in the 'cortisol fraction'. As described in the Methods section, this fraction was in fact analysed further and the relative amounts of cortisone acetate, cortisone and cortisol determined. The total plasma values plotted comprised for the most part cortisone acetate which represented between 50 and 80 % of the concentration; the contribution of cortisol itself was invariably small since it never represented more than 8 % of the total concentration. The total plasma 'cortisol fraction' concentration was, as expected, very high 2–10 hr after injection in both 5-day and 12-day injected animals. Plasma concentration of these steroids fell markedly with time after the injection.

## DISCUSSION

The experiments described in this paper have demonstrated that single injections of large doses of cortisone acetate or corticosterone at either 5 days or 12 days after birth cause profound functional changes in the small intestine of the young rat and also affect both adrenal and body weight.

The results of the S.C. injection of cortisone acetate at 12 days may be compared with those observations of Halliday (1959) which showed that I.P. injections of 1 or 5 mg of this steroid at 10 or 14 days after birth virtually abolished the absorption of antibodies to *Salmonella pullorum* fed 48 hr after injection of the steroids. The disparity in time course between this result and those of present experiments in which complete cessation of PVP uptake did not occur until 3–4 days after cortisone acetate injection may be attributable to the method of administering the hormone or to the differences in the methods of assessing uptake. Indeed, our results accord more closely with those of Clark (1959) in which the steroid was also injected S.C. In that work, changes in the structure of the young rat small intestine indicative of closure and an associated failure to take up fluorescent antibody appeared 48–72 hr after injection and persisted for at least 6 days.

The time course of the process of steroid-induced closure is in fact of considerable importance, since it may shed light on the mode of action of the hormone on the intestine. Clarke & Hardy (1969*b*) demonstrated that, during natural closure in the rat, the time course of the decline in PVP uptake by the terminal small intestine (*ca.* 72 hr) corresponded well with estimates made, using tritiated thymidine, of the time required completely to replace the villous epithelium in the ileum (63 hr). Thus, it seems that at about 18 days after birth in the normal rat, a more mature type of cell unable to take up macromolecules is produced by the crypts of Lieberkühn and that closure occurs as such cells progressively ascend the villus

displacing the older macromolecule-permeable cells. The time course of closure induced precociously by cortisone acetate indicates that a similar progressive process is taking place, although it takes longer to reach completion than in the control rats (Fig. 2, 5–6 day process after injection at 5 days and a 4-day process after injection at 12 days:control 2–3 days). The more protracted time course of the closure process in younger animals was not unexpected, since it is well known that the villous epithelial cell turnover time is longer in suckling rats than in weaned animals (Koldovsky, Sunshine & Kretchmer, 1966).

One hypothesis to account for the action of particular adrenal steroids on the permeability of the new-born intestine to macromolecules would be that the crypts of Lieberkühn produce cells which take up macromolecules until a certain 'threshold' plasma concentration of steroid is attained. Once this threshold is reached the crypts begin to produce cells which are unable to take up macromolecules. In support of this hypothesis is the increase in plasma corticosterone concentrations seen associated with closure in control animals (Fig. 1), and the precocious induction of closure associated with high blood steroid concentrations produced by exogenous corticosterone or cortisone acetate. It is clear that there are differences in the time courses of steroid induced closure which depend upon the steroid used. Thus, closure appears to be permanent after cortisone acetate administration (Fig. 2) and this is associated with a relatively slow fall in blood steroid concentrations such that they are still very high even 6 days after the original injection (Fig. 6). In contrast, the decline in PVP uptake after corticosterone injection is transient (Fig. 4) as indeed are the high blood steroid levels (Fig. 6).

It might appear from these results that the hypothesis that the crypts produce macromolecule-impermeable cells when blood steroid concentrations are above 'threshold' is substantially supported. However, there are two observations which cast doubt upon this simple explanation. First, if one examines histologically the intestine of steroid treated animals (V. G. Daniels & R. N. Hardy, in preparation) the precocious closure promoted by cortisone acetate is, as predicted, associated with cytological changes on the villus, closely comparable with those previously described during normal closure (Clarke & Hardy, 1969*b*). Thus it appears that, as the hypothesis above would require, a new population of macromolecule-impermeable cells comes progressively to occupy the entire villus. However, the transient decline in PVP uptake following corticosterone administration is not accompanied by such changes. Indeed, it is difficult to detect any structural change at all at the light microscope level in such animals. It would appear therefore that corticosterone alters the permeability of the cells once they have occupied the villus, rather than

inducing the formation of a different cell type in the crypts. The second obstacle to the acceptance of the hypothesis that closure is dependent upon blood adrenal steroid concentrations follows from the results of bilateral adrenalectomy (Daniels, Hardy & Malinowska, 1973). For, after this procedure, closure still occurs, although it is delayed by 3–4 days beyond the normal time. Thus it seems that high levels of adrenal steroids are not an absolute requirement for closure, although they may be essential in order for closure to occur at the normal time.

It is well known that exogenous corticosteroids induce a number of changes in the rate of development of the enzyme systems of the suckling rat intestine (Koldovsky, 1969).

It is not clear, however, to what extent the change in macromolecular absorption can be attributed to changes in the activity of specific enzyme systems, although this warrants further investigation.

The amounts of steroids administered during the present experiments were, of course, extremely large and produced totally unphysiological blood concentrations (Fig. 6). Such quantities of steroid are, however, comparable with those used by Halliday (1959) and Clark (1959) in rats and mice, by Payne & Marsh (1962) in pigs, and Gillette & Filkins (1966) in puppies, in which two latter groups adrenal steroids also appeared to promote precocious closure.

In the present experiments, steroid administration produced changes in the rate of growth of the whole animal and in particular the adrenal weight. Administration of both steroids resulted in an impairment of adrenal growth in the 12-day-old rats, but no decrease in adrenal weight occurred at 5 days with either steroid. This is an interesting difference which could be explained on the basis of a failure of increased plasma steroid levels to decrease ACTH secretion in the younger rats.

If this is the explanation of the different response at these two ages it might be concluded that the cells in the intestine develop their responsiveness to these steroids before those at the site of negative feed-back in the hypothalamus and/or the pituitary.

Both corticosterone and cortisone acetate produced a greater depression of total body weight gain when given at 5 days than at 12 days. This is the opposite effect to that noted on adrenal weight. It is possible that at 5 days of age the growth retarding effect of inappropriately large amounts of exogenous steroid are less easily countered by the growth promoting systems. However, an alternative explanation would be that since exogenous steroid does not produce inhibition of ACTH in the 5-day-old group, the total available circulating steroid at this age will be equivalent to the sum of exogenously administered steroid and the endogenously secreted hormone. Since endogenous steroid levels are similar at both ages

(Fig. 1) this would explain the greater loss of weight in the 5-day-old group than in the 12-day group in which the suppression of adrenal growth probably reflects inhibition of ACTH secretion. It should be noted that the increase in endogenous plasma steroids at day 16–20 (Fig. 1) is reflected in a dip in the normal growth curve at this age.

When the two steroids, cortisone acetate and corticosterone, are compared, the effect of cortisone acetate on total body weight is greater at both ages, which could be explained by the more rapid clearance of corticosterone from the blood demonstrated in Fig. 6. Similarly, although neither cortisone acetate nor corticosterone affect the adrenal weight at 5 days, at this age cortisone acetate produces a decrease in total body weight which is more prolonged than corticosterone. Interpretation of these results is difficult since the dose of cortisone acetate given at 5 days was twice as high when expressed per unit body weight as that at 12 days. However, corticosterone administration at the two ages was roughly the same when expressed relative to body weight and in both instances the effect on total body weight is less pronounced than that produced by cortisone acetate.

We are grateful to the Agricultural Research Council for financial support, and to Mr D. Clark and Mr L. Bancroft for their excellent care of the experimental animals.

## REFERENCES

CLARK, S. L. (1959). The ingestion of proteins and colloidal materials by columnar absorptive cells of the small intestine in suckling rats and mice. *J. biophys. biochem. Cytol.* **5**, 41–48.

CLARKE, R. M. & HARDY, R. N. (1969*a*). The use of [$^{125}$I]polyvinyl pyrrolidone (K60) in the quantitative assessment of the uptake of macromolecular substances by the intestine of the young rat. *J. Physiol.* **204**, 113–125.

CLARKE, R. M. & HARDY, R. N. (1969*b*). An analysis of the mechanism of uptake of macromolecular substances by the intestine of the young rat (closure). *J. Physiol.* **204**, 127–134.

DANIELS, V. G. & HARDY, R. N. (1971). The influence of some endocrine organs on the uptake of macromolecules in the new-born rat intestine. *Proc. Int. Union of Physiol. Sciences*, vol. IX, p. 130. Munich: German Physiological Society.

DANIELS, V. G. & HARDY, R. N. (1972). The role of the adrenal gland in the control of intestinal absorption of macromolecules by the young rat. *Experientia* **28**, 272.

DANIELS, V. G., HARDY, R. N., MALINOWSKA, K. W. & NATHANIELSZ, P. W. (1972). Adrenocortical hormones and the absorption of macromolecules by the small intestine of the young rat. *J. Endocr.* **52**, 405–406.

DANIELS, V. G., HARDY, R. N. & MALINOWSKA, K. W. (1973). The effect of adrenalectomy or pharmacological inhibition of adrenocortical function on macromolecule uptake by the new-born rat intestine. *J. Physiol.* **229**, 697–707.

GILLETTE, D. D. & FILKINS, M. (1966). Factors affecting antibody transfer in the new-born puppy. *Am. J. Physiol.* **210**, 419–422.

HALLIDAY, R. (1955). The absorption of antibodies from immune sera by the gut of the young rat. *Proc. R. Soc.* B **143**, 408–413.

HALLIDAY, R. (1959). The effect of steroid hormones on the absorption of antibodies by the young rat. *J. Endocr.* **18**, 56–66.

HELYER, B. J. & HOWIE, J. B. (1963). The thymus and autoimmune diseases. *Lancet* **ii**, 1026–1029.

KOLDOVSKY, O., SUNSHINE, P. & KRETCHMER, N. (1966). Cellular migration of intestinal epithelia in suckling and weaned rats. *Nature, Lond.* **212**, 1389–1390.

KOLDOVSKY, O. (1969). *Development of Functions of the Small Intestine in Mammals and Man.* Basle and New York: S. Karger.

MALINOWSKA, K. W., HARDY, R. N. & NATHANIELSZ, P. W. (1972). Neonatal adrenocortical function and its possible relation to macromolecular uptake by the small intestine of the guinea pig and rabbit. *J. Endocr.* **55**, 397–404.

MURPHY, B. E. P. (1967). Some studies of the protein-binding of steroids and their application to the routine micro- and ultra-micro measurement of various steroids in body fluids by competitive protein-binding radioassay. *J. clin. Endocr. Metab.* **27**, 973–990.

MURPHY, B. E. P. (1971). 'Sephadex' column chromatography as an adjunct to competitive protein binding assays of steroids. *Nature, Lond.* **232**, 21–24.

PAYNE, L. C. & MARSH, C. L. (1962). Gamma globulin absorption in the baby pig: the non-selective absorption of heterologous globulins and factors influencing absorption time. *J. Nutr.* **76**, 151–158.