intervals than these authors, the random nature of the putative episodes should have ensured their detection. Similar studies using the same immunisation schedule also failed to observe episodic release[9].

Each of the antisera obtained showed comparatively high specificity for equilin. In addition, R3 and R4 produced antisera (R3 and R4 B14-18) of reasonable titer. An antiserum raised against equilin-17-(*O*-carboxymethyl)oxime-BSA[10] using the same immunisation procedure was less specific and showed, for example, a cross-reaction of 38% for equilenin[11]. The high specificity of all the present antisera confirms that position 3 is suitable for derivatisation for most oestrogens[6,12,13].

3 of the rabbits consistently produced antisera with similar interactions with equilin sulphate, oestrone and equilenin (table 2). The other rabbit (R3) produced antisera with a higher cross-reaction for equilin sulphate than oestrone. This clearly illustrates the possible variation in animal responses to identical antigens presented in the same manner. Furthermore it is difficult to decide whether the changes in specificity observed from bleed to bleed are the result of non-specific influences or are real changes induced either by changes in circulating antigen or antibody. Antisera cross-reactions have been seen to vary from week to week in an unpredictable fashion in a non-specific anti-testosterone antiserum[14], changes of $\pm 30\%$ being seen in the 5α-dihydrotestosterone cross-reaction. In the present study the variation is observed in antisera of high specificity. Therefore, it would appear that these cross-reactions are due to the production of antibody populations of differing specificity, and that they contain a smaller contribution due to errors in recognition of antigen by antibody than might have been supposed. Such a situation implies that high specificity antisera (as well as poorer antisera) could be further improved by suitable fractionation[15].

Although a previous report[7] showed an increase in specificity during immunisation, our data indicate no relationship between booster antigen injection and bleed specificity. There is also no apparent relationship between titer and specificity. Thus whilst constant monitoring (perhaps even on a daily basis) is required in order to obtain maximum titer using the immunisation procedure described, the situation as regards specificity is less clear.

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Studies on the relationships between biotin and the behaviour of B and T lymphocytes in the guinea-pig

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Summary. In biotin-deficient guinea-pigs the number of circulating neutrophils is increased; lymphocytes carrying B and T markers are decreased. Incubation with biotin increases significantly the number of lymphocytes carrying B and T markers, from biotin-deficient guinea-pigs; no increase was observed when the lymphocytes from normal guinea-pigs were incubated.

The importance of biotin in the defence mechanism of the organism is well known. The data in the literature indicate that in animals deficient in biotin, as with deficiencies of other vitamins[1-3], there is a reduction in the production of antibodies. The administration of biotin to the normal rat increases the immunological reactivity: an increase of hemolysin production in response to inoculation of sheep erythrocytes has been observed[4]; in the case of the splenectomized rat it exerts a protective action in the course of the infection from *Haemobartonella*[5]. Moreover, the administration of biotin to the normal rat produces an increase of the activity of the RES demonstrable either by means of the evaluation of the kinetics of the disappearance of colloidal carbon from the blood stream, or by the assessment of the granulomatous reaction to the inoculation of agar gel in the s.c. tissue[6]. The biotin increases the in vivo and in vitro

Table 1. Circulating leukocytes in normal and biotin-deficient guinea-pigs

Groups	Leukocytes (No./mm^3)	Leukocytes series (%) Lymphocytes	Monocytes	Neutrophils	Eosinophils	Basophils
Normal guinea-pigs (10)	7632 ± 248	62.87 ± 1.41	2.98 ± 0.29	31.63 ± 1.72	1.95 ± 0.29	0.57 ± 0.15
Biotin-deficient guinea-pigs (10)	15384 ± 1561*	33.08 ± 1.21*	2.32 ± 0.20 NS	62.60 ± 1.30*	1.60 ± 0.20 NS	0.40 ± 0.12 NS

Numbers in parentheses indicate the number of animals. Values are expressed as mean ± SE. * $p < 0.001$, significant difference from normal (Student's t-test). NS, no significant difference from normal.

phagocytosis of *Staphylococcus epidermidis* by rat[7] and human[8] neutrophils; this is due either to an increase of the phagocytic index (percentage of neutrophils that have engulfed bacteria) or the avidity index (mean number of bacteria taken into cells engaged in phagocytosis).

Here we report the results of a study carried out in order to investigate in the guinea-pig the effect of biotin deficiency on the number of leukocytes and the frequency of B and T lymphocytes in the blood stream.

Materials and methods. Hartley female guinea-pigs, average initial weight 200–240 g were utilized. Biotin deficiency was obtained by feeding for 7–8 weeks a diet free of biotin and with added avidin[9]. Control animals received the same diet and in addition s.c. injection of an aqueous solution of biotin (500 µg/guinea-pig/week). Following 12-h fasting the animals were anaesthetized with 50 mg/kg b.wt pentobarbital sodium i.p. The blood samples for the preparation of lymphocytes were taken from the heart and collected in a glass tube. To 9.5 ml of blood, 0.5 ml saline with 2.7% EDTA and 130 IU heparin were added. The blood specimens were further diluted 1:4 with saline. 20 ml of the diluted blood were put into a tube (inner diameter 25 mm) and carefully layered on top with the separation mixture consisting of 10 ml of a solution of 9% (w/v) Ficoll 400 (Pharmacia, Uppsala) in distilled water diluted with a solution of 32.8% sodium metrizoate until a density of 1.085 (20 °C). The test tubes were centrifuged at $450 \times g$ for 30 min at room temperature[10]. The mononuclear cells remaining on the top of the separation fluid were carefully collected, suspended in phosphate-buffered saline (PBS) and further centrifuged for 20 min. The cells in the pellet were washed twice in PBS and suspended in PBS (4×10^6 cells/ml) when used to demonstrate B lymphocytes, and in medium RPMI 1640 (GIBCO, New York) (4×10^6 cells/ml) when used to demonstrate T lymphocytes. All glass tubes were siliconized. An aliquot of blood was utilized for the determination of the total leukocyte number and the percentage of the different types of leukocytes.

B and T lymphocytes were recognized according to the method described by Sandberg et al.[11]: B lymphocytes were identified on the basis of their receptors for the activated third component of complement (C_3^1), resulting in rosette formation with erythrocyte-antibody-complement complexes (EAC rosettes)[12]; T lymphocytes were identified on the basis of their ability to form rosettes with rabbit erythrocytes (RE rosettes)[13].

The effect of biotin in vitro on the frequency of rosette-forming lymphocytes was investigated on lymphocytes incubated 15 min at 37 °C in order to stabilize the cells and the medium (PBS) and subsequently given biotin and further incubated 30 min. At the end the cell preparations were washed twice and resuspended in biotin-free PBS, and RE and EAC rosetting were performed.

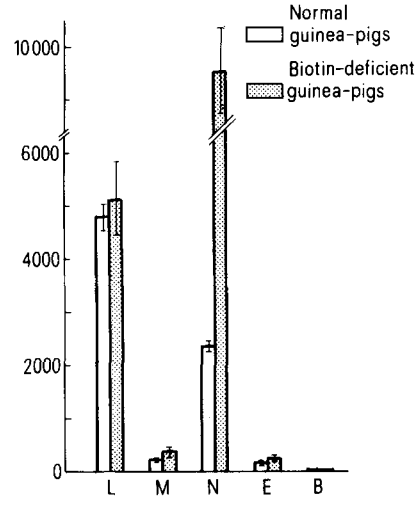
Results and discussion. The data referred to in table 1 agree with those of previous studies[14,15]; in deficient animals an increase in the number of the leukocytes and variations in the percentage of the different types of leukocytes were observed.

The variations in deficient animals were only, or mainly, due to an increase in the number of circulating neutrophils (fig.). The frequency of EAC and RE rosettes in lymphocyte suspensions from blood stream are listed in table 2. The data show that the EAC and RE rosettes were much less frequent in the blood of biotin-deficient than of normal guinea-pigs; that is to say the biotin deficiency induces a

Table 2. Frequency of rosette-forming lymphocytes with RE and EAC in the blood of normal and biotin-deficient guinea-pigs

Source of lymphocytes	No. of experiments	RE rosettes (T lymphocytes)	EAC rosettes (B lymphocytes)
Normal guinea-pigs	10	49.55 ± 1.31%	16.08 ± 0.55%
Biotin-deficient guinea-pigs	9	16.99 ± 0.57%*	8.31 ± 0.73%*

Each experiment was carried out using pooled lymphocyte samples from 2 animals. Values are expressed as mean ± SE. * $p < 0.001$: statistically significant (Student's t-test).



Circulating leukocytes; average values in normal and in biotin-deficient guinea-pigs. Abscissa: lymphocytes (L), monocytes (M), neutrophils (N), eosinophils (E), basophils (B). Ordinate: leukocytes, number per mm^3.

Table 3. Frequency of rosette-forming lymphocytes with RE and EAC in the blood of normal and biotin-deficient guinea-pigs. Cell suspensions incubated in the absence or in the presence of biotin

Source of lymphocytes	No. of experiments	Incubation in the presence of	RE rosettes (T lymphocytes)	EAC rosettes (B lymphocytes)
Normal guinea-pigs	5	PBS	48.44 ± 0.94%	16.16 ± 0.70%
	5	PBS + biotin (20 µg/ml)	49.43 ± 0.37%	16.14 ± 0.83%
	5	PBS + biotin (100 µg/ml)	49.19 ± 1.27%	18.10 ± 0.60%
Biotin-deficient guinea-pigs	5	PBS	16.97 ± 0.82%	8.66 ± 0.97%
	5	PBS + biotin (20 µg/ml)	22.65 ± 1.06%	13.05 ± 0.55%
	5	PBS + biotin (100 µg/ml)	30.00 ± 1.34%	18.67 ± 1.52%

Each experiment was carried out using pooled lymphocyte samples from 2 animals. RE and EAC rosetting were performed following 30 min incubation at 37 °C.

decreased number of lymphocytes carrying B and T markers.

The effect of biotin treatment of peripheral blood lymphocytes on EAC and RE rosette formation is shown in table 3. The RE rosettes in the blood of deficient guinea-pigs were significantly increased when the lymphocytes had been pre-treated with biotin; the number increased following the increase of biotin in the incubation medium (20 µg and 100 µg per ml of lymphocyte suspension): the values come within the range of values shown by normal guinea-pigs. No increase of the RE rosettes was observed when the peripheral blood lymphocytes from normal guinea-pigs were pre-treated with biotin. The percentage of EAC rosettes of biotin-treated lymphocytes was increased when the peripheral blood lymphocytes from either normal or biotin-deficient guinea-pigs were utilized. A slight but statistically significant increase of EAC rosettes was observed when the peripheral blood lymphocytes from normal animals were pre-treated with biotin at a concentration of 100 µg/ml of lymphocyte suspension.

On the lymphocytes of the peripheral blood from biotin-deficient animals the various concentrations of biotin had an enhancing effect on EAC rosette formation. The percentage of EAC rosettes reached the normal values when the lymphocytes were pre-treated with biotin at a concentration of 100 µg/ml of lymphocyte suspension. Such results confirm once again the importance of biotin for the defence mechanisms of the organism: this is probably related to a regulatory activity of biotin on the biochemical pathways[16].

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Effect of starvation on the blood of *Ophiocephalus punctatus* (Bloch)

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Summary. During starvation the values of RBC, WBC counts, Hb content and packed cell volume declined. After 15, 21 and 27 days of starvation little fluctuation was noted but major fluctuation was recorded after 33 days of starvation in all the above parameters.

The physiological response of an animal to fasting is closely related to its nutritional state, which alters from time to time. Starvation affects the normal body metabolism and if it is prolonged may even cause the death of the animal. Decline in the various body constituents of fish, after starvation, have been reported by several authors[2-4]. However, the effect of starvation on the blood parameters of fish has attracted little attention[5-7]. This paper deals with the effect of starvation on the blood of the fresh water fish *Ophiocephalus punctatus* (Bloch). The fish were starved for 33 days.

Material and methods. The fish, *Ophiocephalus punctatus* were procured from the local fish market during the month of November, 1979 and were kept in aquaria for acclimation for 7 days. After feeding daily for 7 days the fish were starved. Blood samples were collected after 15, 21, 27 and 33 days. The blood was obtained from the caudal vein and transferred into double oxalated vials. For the total RBC and WBC counts a haemocytometer was used. Haemoglobin (Hb) was estimated by Sahle's haemometer. The ultra micro-method as described by Natelson[8] was applied for the estimation of packed cell volume (PCV). Blood samples collected from normally-fed fish served as controls.

Observations. In the present study notable variations in the total RBC and WBC counts, Hb content and PCV of the starved fish were observed. The normal values of these in the control fish were: RBC, 3.31×10^6/mm^3; WBC, 13000/mm^3; Hb, 15.0 g%, and PCV, 49.23%. A direct correlation was found between the values of the above parameters and periods of starvation, i.e. values gradually decreased with the increasing period of starvation (table). The values declined gradually in the beginning, i.e. after 15, 21 and 27 days but a sudden fall was observed after 33 days of starvation (table).

Discussion and conclusions. In the present study the values of RBC and WBC counts, Hb content and PCV decreased with an increase of the starvation period. The values showed a gradual decline of all parameters during a period of 27 days of starvation but a sudden fall was observed after 33 days of starvation (table).

Smallwood[9] observed that the red blood cell count of *Amia calva* dropped from 1640000/mm^3 to 400000/mm^3 after starvation for 20 months. Murachi[10] found a decrease in PCV from 50% to 30% and a corresponding reduction in Hb from 11 to 7 g% in *Cyprinus carpio.* Higginbotham and Meyer[6] also observed a fall in PCV and Hb content in *Ictalurus lacustris punctatus,* when fishes were in poor condition (emaciated). Thus, it may be suggested that with a variable period of starvation the RBC count and Hb content also decrease accordingly.