vening shoulder region, were irradiated. Specimens were then amputated through both forearms and kept in 25 IU/ml vitamin A for 15 days at room temperature. Irradiated forearm stumps did not regenerate even after vitamin treatment (table), thus providing no evidence of cellular migration from the shielded upper arm. Shielded forearm stumps regenerated a virtually complete arm, containing a partial or entire repeat of the stump humerus (fig. d). Since all proximal tissue had been irradiated and thus rendered incapable of regeneration, this result demonstrates that local forearm cells have been respecified to attain the status of upper arm cells.

We used retinol palmitate because it is available in a relatively stabilized form which can be accumulated by storage in the liver and thus metabolized less rapidly than retinoic acid[7]. Our treatment caused a temporary reduction of appetite and growth, and the regenerates contained a variable amount of structural repetition. Higher concentrations of retinol palmitate produce more extreme responses[2], but also increase the incidence of limbs whose regeneration is greatly delayed or inhibited. The delay of regeneration during treatment is not responsible for the serial repetition. Even greater delays resulting from higher doses tend to result in typical regenerates, as does the delay caused by repeated denervation[8]. Reamputated limbs also regenerate typically, indicating that excess retinoids do not persist very long in the body and do not have a permanent effect on mature tissues. We conclude that vitamin A affects cells close to the site of amputation, probably after they have dedifferentiated to form blastemal mesenchyme, by respecifying them to adopt a more proximal character.

Types of regenerate scored 6 weeks after forearm amputation

Pretreatment	Vitamin A IU/ml	Regenerated structure None	Typical	Excessive
Normal arms	none	0	12	0
Reversed arms	none	0	12	0
Normal arms	25	6	0	6
Reversed arms	25	2	4	6
Forearms X-rayed	25	12	0	0
Upper arms X-rayed	25	0	0	12

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Occurrence of polyploidy and multinuclearity in the differentiating liver of chick embryo

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Summary. Increase in nuclear size in liver has been used as an index of polyploidy. It has long been considered that the occurrence of polyploidy and multinuclearity are characteristics of mammalian liver. The present study shows the occurrence of these phenomena in the liver of birds, so these features are not confined to mammals. 3 classes of nuclear size groups have been identified. The simultaneous occurrence of polyploidy and binuclearity indicates some sort of interrelationship between them.
Key words. Chick embryos; liver, chick; chick, liver; nuclear size; polyploidy; multinuclearity.

Differences of nuclear size in the hepatic parenchyma of mammals have been observed by several investigators[1,2]. Nuclear size has frequently been used as an index of ploidy by investigators working with hepatic tissue. Several authors[3-12] have shown that doubling of the DNA content in liver nuclei is indeed reflected by roughly a doubling of the volume. Jacoby[1] concludes that the higher nuclear class or polyploid classes are characteristic of mammalian liver, and are absent in any other groups of vertebrates.

In addition to the polyploid nuclei, liver cells frequently contain more than 1 nucleus, binuclearity being the most common type of multinucleated condition. Munzer[13] reported that there are wide species differences in the frequency of binucleated cells, and also noted that they are few in the new born and appear with greater frequency in adults.

Most work on liver polyploidy and binuclearity has been carried out in mammals, but practically no work have been done in this regard on avian liver. Medda and Shamsuddin[14] observed that up to the 12th day of incubation, the increase and decline of DNA content per unit weight of liver tissue run parallel with the increase and decline of mitotic index, but after the 12th day there is a decrease in mitotic index and an increase in the DNA content. The high DNA content, in spite of the lower rate of cell proliferation, after the 12th day of incubation, led them to speculate about the development of polyploidy and binuclearity in chick liver cells. However, only direct microscopical observation could confirm the above speculation. For this reason, in the present work it was decided to study the percentage of multinucleated cells and the average volume of the nuclei, which may be correlated with the degree of ploidy, at different developmental stages of liver in chick.

Materials and methods. The materials were the eggs and embryos of white Leghorn chicks obtained from Govt. Poultry Farm, Burdwan, W. Bengal. The eggs were incubated at 38 °C, with 75% relative humidity.

Since EDTA has been extensively used as a cell-separating detergent[15-17] we used EDTA solution for separting cells. By trial and error methods we devised the following procedure for separating liver cells. The livers of the embryos were dissected out quickly, cut into small pieces and then placed in a dissociating mixture (equal volumes of 0.015 M EDTA solution and 0.22 M NaCl solutions) in the proportion of 2 ml per 100 mg of tissue, along with some glass beads, and shaken for half an hour. The entire mass was then passed through a fine-meshed silk to remove the glass beads and tissue clumps. The dissociated cells in solution were fixed by 0.01 ml formalin/ml of dissociating mixture, and shaken for 5 min. After fixation samples were centrifuged and the supernatant discarded. The fixed

cells in the residue were washed with distilled water several times to remove the dissociating mixture and the formalin. The cells were then suspended in a small volume of distilled water. One drop of suspended cells was placed on a clean slide, and a drop of 1% toluidine blue was added to it for staining. A cover glass was placed on it and then sealed by a sealing material. From these slides, nuclear volumes and multinucleated cells were studied. Average nuclear volumes were determined from the average diameters of the liver parenchymal cells by using the formula $V^4/_3\ \pi r^3$, where V is the volume and r is the measurement of the radius.

Results and discussion. A steady increase in the mean value of the nuclear volume of chick liver cells was observed from the 8th to the 16th day of incubation, when the value was highest; it then declined on the 18th and 20th day of incubation. In table 1, the figure in each stage represents the mean value of nuclear volume of at least 300 cells. From the observations, at least 3 classes of nuclear size were identified. The mean nuclear size belonging to the first class is approximately 64 μm^3, and those of the second and the third classes are 127.88 μm^3 and 220.98 μm^3 respectively (table 2). It should be noted that the nuclear size of each higher class is more or less double of the preceding lower class. Since in the case of mammalian liver, nuclear size has been used an index of ploidy, it may be suggested that the larger nuclear volume in chick liver cells is due to the occurrence of polyploidy. Again, in the early stages of developing liver, most of the nuclei belong to the first class, so it may be considered that this class consists of diploid nuclei (2 n). Similarly the second and the third class consists of tetraploid (4 n) and octaploid (8 n) nuclei. Table 2 shows that the higher nuclear size groups started to appear on the 10th day and their percentages gradually reach a maximum on the 16th day of incubation with a concomitant decline of the diploid nuclei i.e. the nuclei of the first class. After the 16th day the percentage of diploid nuclei is increased with the concimitant decrease of the higher nuclear size groups. Thus it may be suggested that, in chick liver, polyploidization started on day 10, attained a maximum on the 16th day of incubation, and then declined.

Among the multinucleated cells, binuclearity is most prevalent. Binucleated cells first appeared on the 10th day, trinucleate cells appeared on the 14th day, and the percentages of both types of cells reached a maximum on the 16th day of incubation (table 3), and then declined. The present investigation is fully in agreement with the view of Medda and Shamsuddin[14] who suggest that after the 12th day of incubation, the lower rate of mitotic index but higher contents of DNA is due to the occurrence of polyploidy and binuclearity during this period. In animals, polyploidy generally occurs in those organs which have very high synthetic and secretory activity, e.g. salivary glands of Dipteran larvae, silk glands etc. From the point of view of secretion and absorption a large cell should be more effective than the same mass of smaller cells[18]. Our previous studies on the growth of chick liver show that there is enormous increase in cell size and accumulation of carbohydrates, lipids etc. between the 16th and 20th days of incubation, indicating the hyperactivity of chick liver cells. The occurrence of polyploidy and binuclearity between the 10th and 16th days may be regarded as a preparation for the hyperactivity. This is in agreement with the view of Doljansky[19] who considered the polyploidy and binuclearity as a further step in differentiation when the hepatic cells become functionally more efficient. The partial decline of these 2 processes after the 16th day may be partly due to the increase of cell number as mitosis occurs, although the rate becomes lower at a later period, throughout the entire period of incubation[20], or the rates of the processes of polyploidization and development of binuclearity themselves become low.

Thus it becomes increasingly clear that, in hepatic cells, the occurrence of polyploidy and binuclearity are not a mammalian monopoly; they also occur in birds. The difference is that the chick liver attains polyploidy and binuclearity during the embryonic stage, whereas in mammals, these processes occur during postnatal development. A certain percentage of polyploid nuclei has also been reported in some amphibian species by Bachman and Cowden[21], Goin et al.[22] and Bachman et al.[23]. Thus it is erroneous to suggest a virtual absence of polyploidy and binuclearity in liver of other vertebrates, except mammals. The simultaneous occurrence of polyploidy and binuclearity may be due to the presence of some sort of interrelationship between them. In agreement with Beams and King[24], Wilson and Leduc[25] concluded that binuclearity arise by failure of cytoplasmic division, and polyploidy arises from binucleated cells by endomitosis. The present study reveals that the increase of nuclear size occurs while binuclearity is still very high, which may corroborate the view that polyploid cells originate from binucleate cells.

Table 1. Average volume of the nucleus in developing chick liver with standard error

Age in days	Mean volume of the nucleus (in μm^3)
8	54.130 ± 2.09
10	64.373 ± 4.26
12	102.472 ± 3.95
14	105.591 ± 4.13
16	126.647 ± 4.82
18	92.952 ± 2.63
20	83.796 ± 2.17

Table 2. Frequencies of different classes of nuclei in liver of chick embryo of different ages

Age in days	Percent of different classes of nuclei		
	(2 N) 64 μm^3	(4 N) 127.88 μm^3	(8 N) 220.98 μm^3
8	100.00	–	–
10	88.00	8.67	3.33
12	84.66	25.00	10.34
14	60.00	21.67	18.33
16	43.00	42.67	14.33
18	60.00	33.33	6.67
20	62.00	35.67	3.33

Table 3. Proportion of uninucleate, binucleate and trinucleate cells in developing liver of chick embryo

Age in days	Total number of cells counted	Number of uninucleate cells	Number of binucleate cells	% of binucleate cells	Number of trinucleate cells	% of trinucleate cells
8	100	100	–	–	–	–
10	300	292	8	2.66	–	–
12	290	273	17	5.76	–	–
14	300	263	35	11.66	2	0.66
16	315	259	49	15.55	7	2.22
18	300	262	36	12	2	0.66
20	355	325	29	9.03	1	0.003

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Periimplantation-like growth and development of mouse blastocysts in medium containing horse serum[1]

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Summary. Horse serum (HS) supported growth and differentiation of blastocysts as well as or better than fetal bovine serum (FBS) ($p < 0.01$) as measured by a) the onset of trophoblastic outgrowth, b) the size of the resultant outgrowths, c) the size of nuclei in trophoblasts after their outgrowth and d) the size of the inner cell mass in outgrowths. Moreover, polyamines were found to be approximately 10–100 times less toxic to embryos when added to medium containing HS than when the medium contained FBS.
Key words. Mouse blastocysts; culture medium; horse serum; polyamine synthesis; trophoblastic outgrowths.

We describe here the use of horse serum (HS) as an alternative to fetal bovine serum (FBS) as a component of medium used to grow periimplantation mouse blastocysts through the stage of trophoblastic outgrowth in vitro. The developmental events which occur during culture of these blastocysts are analogous to periimplantation events which occur in utero[2,3]. Thus, this culture system is useful for examining the biochemical mechanism of nidation, at least as it relates to the blastocyst. Moreover, when examining biochemical events associated with periimplantation development in vitro it is sometimes necessary to add enzyme inhibitors, substrates and or products to the culture medium[4–7]. Because serum contains enzymes and other biochemicals it is possible that substances added to the medium may interact with components of the serum in the medium. For example, cleavage-stage embryos[4] and blastocysts[5] appear to be growth-inhibited[5] and/or killed[4,5] when spermine an/or spermidine (polyamines) are added to medium containing bovine serum albumin[4] or FBS[5]. Presumably, the added polyamines are oxidized by polyamine oxidase present in FBS[8,9] and bovine serum albumin preparations[10]. As is the case for other cell types[8,9,11], the oxidation products of polyamines appear to be particularly inhibitory and/or toxic to the cells of blastocysts[5]. While these toxic substances are produced from polyamines in the presence of bovine sera and products of bovine sera[8–10], the same toxic effect has not been observed for other cell types when HS is substituted for FBS[8,9]. Thus we examined the effect of polyamines on the growth and development of mouse blastocysts in vitro in medium containing HS instead of FBS.

Materials and methods. Swiss, ICR mice (Harlan, Sprague Dawley, Inc.) which had been induced to ovulate and mate, were ovariectomized before 12.00 h on the 4th day post coitus

Effect of serum type and concentration on outgrowth formation by 'delayed implanting' mouse blastocysts cultured in Dulbecco's modified Eagle medium

Parameter measured and duration of culture[b]	Source, type and concentration of serum[a]									
	Grand Island Biological Co.				HyClone (Logan, Utah)					
	Horse serum		Fetal bovine		Horse serum		Fetal bovine$_1$		Fetal bovine$_2$	
	3%	10%	3%	10%	3%	10%	3%	10%	3%	10%
Percentage of outgrowths after 42 h	91.1 (45)	97.7 (44)	28.9 (45)	58.7 (47)	21.7 (69)	50.0 (66)	34.4 (64)	38.1 (63)	37.3 (55)	47.6 (63)
Diameter of outgrowths (μm) after 70 h (mean ± SE)	316 ± 19 (15)	391 ± 17 (20)	306 ± 17 (20)	365 ± 17 (20)	276 ± 15 (25)	357 ± 15 (25)	304 ± 15 (25)	342 ± 15 (25)	287 ± 15 (25)	325 ± 17 (20)
Diameter of trophoblastic nuclei (μm) after 70 h (mean ± SE)	22.6 ± 1.6 (20)	25.6 ± 1.6 (20)	22.3 ± 1.6 (20)	27.0 ± 1.6 (20)	21.2 ± 1.4 (25)	23.5 ± 1.4 (25)	23.5 ± 1.4 (25)	27.5 ± 1.4 (25)	23.0 ± 1.4 (25)	23.0 ± 1.4 (25)
Diameter of inner cell mass (μm) after 70 h (mean ± SE)	97.1 ± 7.0 (17)	103.6 ± 7.0 (17)	113.7 ± 7.0 (17)	119.1 ± 7.0 (17)	114.9 ± 6.3 (22)	121.7 ± 6.3 (22)	100.1 ± 6.3 (22)	124.6 ± 6.3 (22)	106.9 ± 6.3 (22)	110.2 ± 6.3 (22)

[a] The total number of blastocysts cultured (1st row) or measurements made (last 3 rows) is shown in parentheses. Pooled data from 4 or 5 experiments are presented. [b] All parameters had larger values at higher serum concentrations ($p < 0.01$ for rows 1, 2 and 3; $p < 0.05$ for row 4). Horse serum from Grand Island Biological Co. produced more rapid onset of outgrowth than other sera ($p < 0.01$). No other statistically significant differences were observed.