Changes in the Chemical Composition and the Enzymic Activities of Hepatic Microsomes of the Chick Embryo during Development

BY J. K. POLLAK AND DENISE B. WARD*

*Department of Histology and Embryology and Department of Biochemistry,
University of Sydney, Sydney, New South Wales, Australia*

(Received 20 September 1966)

1. The values of the protein, RNA and phospholipid concentrations within the total microsomal fractions obtained from different stages of embryonic chick liver are compared. 2. Only the phospholipid content increases significantly with increasing developmental age. 3. The lack of membranes in the early stages of development and the relative constancy of RNA values during development suggests that some of the protein present at the early developmental stages is of a non-membranous non-ribosomal nature. 4. Glucose 6-phosphatase, adenosine triphosphatase, NADH₂-cytochrome *c* reductase and diaphorase all increased in activity as development progressed. 5. Comparisons of submicrosomal fractions with respect to their protein, RNA and phospholipid content showed that in all embryonic stages fraction II (rough-membrane fraction) contained more than 60% of the proteins, RNA and phospholipid of the microsomal fraction. 6. Glucose 6-phosphatase was shown to be present predominantly in fraction II, whereas adenosine triphosphatase was present predominantly in fraction Iab (smooth-membrane fraction). 7. The significance of the differences between the smooth- and rough-microsomal fractions is discussed.

The chemical composition and the enzymic activities of hepatic microsomes of various experimental animals have been studied repeatedly (for references see reviews by Dallner, 1963; Moulé, 1964; Rothschild, 1963; Siekevitz, 1963). However, only in isolated instances have the changes in the ultrastructure of the endoplasmic reticulum during embryonic development been correlated with the changes in the chemical composition and enzymic activity (Caquoin-Carnot & Roux, 1960; Dallner, Siekevitz & Palade, 1966*a,b*; Duck-Chong, Pollak & North, 1964; Pollak & Shorey, 1964*a*). Even here, in the three first-named references, which were concerned with the developing rat foetus, the studies were confined to investigating the changes in the ultrastructure and metabolism for several days before and after parturition.

In a preliminary communication (Pollak & Shorey, 1964*a*) we attempted to correlate changes in the chemical composition and enzymic activity of microsomal fractions with the developmental age of the embryonic chick liver. The present investigation represents a more extensive and enlarged study of this problem. As pointed out previously (Pollak, 1965) embryonic chick liver is

a suitable tissue to use for the study of the formation of endoplasmic reticulum, since in the very early stages of liver development (5 and 6 days) either no or little endoplasmic reticulum is to be observed. As the development of the liver proceeds, more and more endoplasmic reticulum is formed. This system is therefore well suited to an investigation into the causal connexion, if any, between the membranes of the endoplasmic reticulum and morphogenetic changes.

MATERIALS AND METHODS

Fertile eggs of an inbred White Leghorn strain were obtained from the N.S.W. Government Experimental Station (Seven Hills, New South Wales, Australia). Unless otherwise stated these eggs were the source of the embryonic liver used; however, in some of the later experiments White Leghorn × Australorp crossbred eggs were used, as indicated in the text. The eggs were incubated at 38–39° and the developmental stages of the embryos after incubation for a given time were checked by reference to the illustrations and measurements of the Hamburger stages (Lillie, 1952). The chick livers were dissected out and were pooled to obtain the amount of tissue required. In experiments with 6-day-old embryonic liver 60–200 mg. of tissue was used (50–150 embryonic livers), and for experiments with the liver of other embryonic ages approx. 500 mg. was used. All reagents were of analytical grade.

* Present address: Lister Institute of Preventive Medicine Chelsea Bridge Road, London, S.W. 1.

Disodium glucose 6-phosphate was obtained from the Nutritional Biochemicals Corp. (Cleveland, Ohio, U.S.A.) and from the Sigma Chemical Co. (St Louis, Mo., U.S.A.); disodium ATP was from Pabst Laboratories Inc. (Milwaukee, Wis., U.S.A.); NADH₂ was from the Sigma Chemical Co. Cytochrome *c* was prepared by the method of Keilin & Hartree (1945) to an iron content of 0.34%.

The excised chick embryos were washed in cold 0.25M-sucrose, and then the livers were removed and placed into cold homogenizing medium. All subsequent preparative procedures were carried out in a cold room at 0–2°. The livers were blotted dry with Whatman no. 50 filter paper and weighed; the tissue was then homogenized in a tightly fitting 10 ml. glass-Teflon homogenizer (A. H. Thomas Co., Philadelphia, Pa., U.S.A.) with no more than 8 up-and-down strokes at 1400 rev./min. The homogenizing medium was 0.25M-sucrose–1mM-MgCl₂ for the preparation of total microsomal fractions; whenever the Dallner (1963) method for subfractionation was used the MgCl₂ was omitted. The homogenate contained about 50mg. of tissue/ml. Each homogenate was checked by phase-contrast microscopy for

proper dispersion and absence of whole cells. All fractionation procedures were also followed and controlled at every stage by phase-contrast microscopy, and at certain stages by electron microscopy.

Preparation of total microsomal fractions. The method of the preparation of the total microsomal fraction was a modification of that of Ernster, Siekevitz & Palade (1962). The homogenate was centrifuged for 20 min. at 10000g_{av.} in a Beckman–Spinco model L or L2 centrifuge with the SW39L swing-out head. For most tissues 10 min. at 10000g is assumed to be sufficient to remove the nuclear and mitochondrial fractions, but in the present investigation with embryonic chick liver it was demonstrated by electron microscopy that the resulting supernatant still contained a significant number of mitochondria. On prolonging the centrifugation procedure at 10000g_{av.} to 20 min., the supernatant was free from mitochondria, and yet the discarded pellet was practically free of microsomal fragments. After the removal of the nuclear and mitochondrial fraction in this manner, the supernatant was centrifuged for 60 min. at 109000g_{av.} in the SW39L rotor

Scheme 1. Flowsheet of fractionation of rough- and smooth-microsomal subfractions.

and yielded the microsomal pellet that was used for chemical and enzymic assays. The pellet was rinsed twice with 0.25 M-sucrose-1 mM-MgCl₂ and then resuspended in the same solution by hand homogenization, so that the final suspension contained from 1-5 mg. of protein/ml.

Preparation of microsomal subfractions. Three microsomal subfractions (Scheme 1) were obtained by a modification of the sucrose-CsCl-MgCl₂-discontinuous-gradient method of Dallner (1963). This method yields three distinct fractions. One is a rough-membrane fraction (fraction II) and the other two are smooth-membrane fractions (fractions Ia and Ib). Since the presence of Mg²⁺ in the initial stages of the fractionation procedure would prevent the separation of fractions II and Ib, MgCl₂ was omitted from the initial stages of the preparation. The nuclei and mitochondria were sedimented at 10000g_{av} for 20 min. as for the preparation of the total microsomal fraction. The supernatant was carefully pipetted off and M-CsCl was added to a final concentration of 15 mM; 3 ml. of this suspension was then layered over 1.8 ml. of 1.3 M-sucrose-15 mM-CsCl and centrifuged at 109000g_{av} for 120 min. After Dallner's (1963) nomenclature the resulting pellet was named fraction II. The upper layer and the layer at the interface of the two sucrose layers were removed and M-MgCl₂ was added to a final concentration of 10 mM. Of this suspension 3 ml. was layered over 1.8 ml. of 1.3 M-sucrose-10 mM-MgCl₂ and centrifuged at 109000g_{av} for 60 min. The resulting pellet was called fraction Ib; the upper layer and the layer at the interface were collected and centrifuged at 109000g_{av} for 90 min. and the resulting pellet was called fraction Ia, again after Dallner's (1963) nomenclature. In the experiments that follow enzymic activities of rough and smooth membranes during development, fraction II was equated to the rough-membrane fraction, and the combined fractions Ia and Ib were equated to the smooth-membrane fraction. The latter was obtained by collecting the upper layer and the layer at the interface after the pellet of fraction II was sedimented, and centrifuging these, without any addition of MgCl₂ or further layering, at 109000g_{av} for 90 min.

In the initial experiments all centrifugations were carried out in the SW 39L rotor. In some of the later experiments the 50 angle-head rotor was used; the volumes were altered in proportion and identical results were obtained.

Analytical methods. Protein, RNA and phospholipids were isolated and determined as described by Pollak & Shorey (1964b), except that in some of the later experiments phosphate was determined by the method of Chen, Toribara & Warner (1956).

Enzyme assays. Glucose 6-phosphatase (D-glucose 6-phosphate phosphohydrolase, EC 3.1.3.9) was assayed by the method of Swanson (1955), with an incubation time of 20 min. at 30°. β -Glycerophosphatase (EC 3.1.3.1) activity was determined under essentially the same conditions as glucose 6-phosphatase. The reaction mixture contained 20 mM-sodium β -glycerophosphate and 60 mM-tris-maleate buffer, adjusted to pH 6.5 with 0.2 N-NaOH. Incubation was carried out for 20 min. at 30°. ATPase* (EC 3.6.1.4) was assayed by the method of Ernster *et al.* (1962) except that a final sucrose concentration of 0.09 M was used. In all cases the reaction was stopped with HClO₄, and, after centrifuging off the precipitated proteins, portions of the supernatant suspensions were assayed for

inorganic phosphate by the method of Allen (1940). The assay system for the ATPase reaction was kept in the cold until the addition of the ammonium molybdate reagent, and thus very low zero-time values were obtained.

NADH₂-cytochrome *c* reductase (EC 1.6.2.1) was assayed by the method described by Ernster *et al.* (1962). Readings were taken at 30 sec. and then at 1 min. intervals for 4 min. at 550 m μ with a Unicam SP.600 spectrophotometer. The temperature was maintained at 30 $\pm$ 1° and a 1 cm. light-path was used. The initial rates of the reaction (i.e. within the first 2 min.) were used to compute the results. In nearly all instances the reaction rate was linear up to 4 min. Diaphorase (EC 1.6.4.3) was assayed with 2,6-dichlorophenol-indophenol as terminal electron acceptor at a final concentration of 0.04 mM, under the same conditions as were given for NADH₂-cytochrome *c* reductase, but the addition of KCN was not essential.

All enzyme assays were carried out in duplicate. All assays for enzyme activity were completed within 3 hr. of the preparation of the microsomal suspensions.

Electron microscopy. All microsomal fractions and subfractions were fixed as pellets in buffered 1% osmium tetroxide for 16 hr. at 0-5°. Dehydration and embedding were carried out by the conventional methods with Araldite as an embedding medium. Sections were cut on an LKB Produktor (Stockholm, Sweden) ultra-microtome with glass knives, and were mounted on nitrocellulose-carbon-coated grids. In some instances sections were stained on the grid with a drop of 4% uranyl acetate for 30 min.

RESULTS

The values of the protein, RNA and phospholipid concentrations within the total microsomal fractions obtained from different stages of embryonic chick liver, together with the liver weights at the corresponding developmental stages, are presented in Table 1. The general trend observed is that the protein content of the microsomal fraction isolated from 1 g. of embryonic chick liver remains fairly constant with increasing developmental age, whereas the bound phospholipid content of the microsomal fraction more than doubles between the developmental ages of 6 and 16 days. Microsomal RNA decreases slightly with increasing developmental age. This slight decrease was also previously demonstrated with respect to total cytoplasmic and nuclear RNA (Duck-Chong *et al.* 1964).

Orientated microsomal pellets taken from various developmental stages demonstrated obvious differences. At the 6-day-old stage almost all of the osmiophilic granules (presumed to be ribosomes) were free and not attached to membranes and thus practically no typical rough microsomal vesicles were observed; however, some smooth microsomal vesicles were present (Plate 1). These represented either elements of the smooth endoplasmic reticulum or vesicles derived from the disruption of plasma and nuclear membranes. As the developmental age increased the typical rough microsomal vesicles

* Abbreviation: ATPase, adenosine triphosphatase.

EXPLANATION OF PLATE I

(a) Microsomal fraction from 6-day-old embryonic chick liver fixed in buffered osmium tetroxide and stained with uranyl acetate. The ribosomes are predominantly free and only few smooth membranes are present. Magnification $\times 45\,000$. (b) Microsomal fraction from 16-day-old embryonic chick liver fixed in buffered osmium tetroxide and stained with uranyl acetate. Ribosomes are predominantly bound to membranes, and the microsomal fraction consists of tightly packed smooth and rough membranes. Magnification $\times 45\,000$.

EXPLANATION OF PLATE 2

All the microsomal subfractions were fixed with buffered osmium tetroxide and stained with uranyl acetate. All magnifications $\times 36000$. (a) Fraction Ia from 6-day-old embryonic liver; (b) fraction Ib from 6-day-old embryonic liver; (c) fraction II from 6-day-old embryonic liver; (d) fraction Ia from 12-day-old embryonic liver; (e) fraction Ib from 12-day-old embryonic liver; (f) fraction II from 12-day-old embryonic liver. c.o.g., Clusters of osmiophilic granules.

Table 1. *Liver weights and protein RNA and phospholipid concentrations within the total microsomal fraction of different stages of embryonic chick liver*

Details of the preparations of the 'total microsomal fraction' and of the assay methods are presented in the Materials and Methods section. The results represent mean values $\pm$ s.e.m. whenever the mean is derived from more than four experiments. The number of experiments from which the mean is derived is given in parenthesis. The results refer to values obtained for the microsomal fraction obtained from 1 g. of liver tissue. Since a number of livers were pooled for each experiment and only the total liver weight was determined, no meaningful s.e.m. can be given and thus only the total number of livers from which the mean was derived is given in parenthesis.

Embryo age (days)	Liver wt. (mg.)	Protein (mg./g. of liver)	RNA (mg./g. of liver)	Phospholipid (mg./g. of liver)	Protein/RNA ratio	Protein/phospholipid ratio
6	2.1 (908)	12.8 $\pm$ 1.79 (11)	3.8 $\pm$ 0.31 (10)	2.7 $\pm$ 0.39 (9)	3.4	4.9
7	6.7 (209)	19.1 (4)	3.8 (4)	3.8 (4)	5.0	5.0
8	12.2 (142)	15.6 (3)	3.1 (4)	4.3 (4)	5.0	3.6
9	19.1 (587)	15.6 $\pm$ 0.92 (16)	3.0 $\pm$ 0.20 (15)	3.4 $\pm$ 0.20 (14)	5.2	4.6
10	29.4 (72)	13.9 (2)	3.5 (2)	3.0 (2)	4.0	4.6
12	73.0 (147)	13.9 $\pm$ 0.95 (9)	3.4 $\pm$ 0.12 (8)	3.3 $\pm$ 0.19 (8)	4.1	4.2
16	299.0 (49)	14.6 $\pm$ 0.85 (8)	3.0 $\pm$ 0.18 (8)	5.7 $\pm$ 0.18 (6)	4.9	2.6

became more frequent, and fewer free osmiophilic granules were observed in sections of the microsomal pellet. This is best exemplified by a micrograph of a section of a microsomal pellet derived from 16-day-old embryonic chick liver (Plate 2).

This early paucity of membranes and the increase of membranous structures of the endoplasmic reticulum is difficult to correlate with the relative constancy of protein values during development and also with the high and relatively constant protein/RNA ratios. It has been repeatedly demonstrated that the protein/RNA ratio of ribosomes is about 1 (Hess & Lagg, 1963), and yet microsomal preparations from early embryonic stages that contain only a minimum amount of membranous material and are made up almost entirely of osmiophilic granules (Plate 1) have protein/RNA ratios greatly in excess of 1. While between the developmental ages 6 and 16 days the protein/phospholipid ratio is nearly halved, owing to the increase of bound phospholipid in the microsomal fraction, the protein/RNA ratio increases slightly between 7 and 16 days, and only between 6 and 7 days is there a significant increase in the ratio. These results seem to imply that during the early stages of development proteins of other than ribosomal or membrane origin are present in the microsomal fraction.

The specific activities of all the enzymes assayed increased with progressive development of embryonic chick liver (Table 2). The glucose 6-phosphatase of the microsomal fraction from 6-day embryonic liver was always low, and in two instances could not be detected; nevertheless, we consider that the low mean value does indicate true activity. This is particularly so if one considers that no phosphatase activity at all was

encountered when β -glycerophosphate was used as substrate at pH 6.5. This substrate and this particular pH value were chosen to establish that the phosphatase activity measured at pH 6.5 in the presence of glucose 6-phosphate was true glucose 6-phosphatase activity and not due to a non-specific phosphatase. The activity of glucose 6-phosphatase, which is thought to be a membrane-bound enzyme (see Dallner, 1963), is thus shown to be correlated with the development of microsomal membranes. The same can also be stated about the ATPase activity, which also increases between the developmental ages of 6 and 16 days though the percentage increase in activity is smaller than that of glucose 6-phosphatase (Table 2). It is noteworthy that considerable activity of the latter enzyme is present in the microsomes from 6-day-old embryonic chick liver, in spite of the fact that very few membranous components of the endoplasmic reticulum are observed at this stage. The large standard errors of the ATPase results from the 6-day-old embryonic chick liver indicate the considerable variation of enzyme activity which was encountered.

The two oxidative enzymes, NADH₂-cytochrome c reductase and diaphorase, showed a similar trend to the two phosphatases, namely they increased over the developmental period of 6-16 days (Table 2). As a check on the microsomal origin of the NADH₂-oxidizing enzymes, succinate was added at the end of the experiments to the reaction mixture and in no instance did this cause the reduction of cytochrome c or 2,4-dichlorophenol-indophenol. This indicated the absence of succinoxidase and succinate-dehydrogenase activity, and excluded the likelihood of mitochondrial enzymes being responsible for the oxidation of NADH₂. On

Table 2. *Changes in enzymic activities in microsomes of embryonic chick liver during development*

Details of the microsomal preparation and assay methods are presented in the Materials and Methods section. The results represent mean values $\pm$ s.e.m. The number of experiments from which the mean was derived is given in parenthesis. All incubations were carried out at 30°. The incubation mixture of the glucose 6-phosphatase assay contained 10 μ moles of glucose 6-phosphate, 30 μ moles of maleate buffer, pH 6.5, and 0.1 ml. of microsomal suspension in a total volume of 0.5 ml. The ATPase assay was carried out in the presence of 10 μ moles of ATP, 100 μ moles of tris buffer, pH 7.5, 180 μ moles of sucrose, 8 μ moles of $MgCl_2$ and 0.1 ml. of microsomal suspension in a total volume of 2 ml. NADH₂-cytochrome *c* reductase was assayed in a total volume of 3 ml. containing 0.15 μ mole of cytochrome *c*, 0.3 μ mole of NADH₂, 150 μ moles of tris buffer, pH 7.5, 1.0 μ mole of KCN, 500 μ moles of sucrose and 0.02 ml. of microsomal suspension. Diaphorase was also assayed in a total volume of 3 ml. containing 0.12 μ mole of 2,6-dichlorophenol-indophenol, 0.3 μ mole of NADH₂, 150 μ moles of tris buffer, pH 7.5, 650 μ moles of sucrose and 0.1 ml. of microsomal suspension. The extinction coefficient for 2,6-dichlorophenol-indophenol at pH 7.5 was taken as $21.2 \times 10^{-3} \text{ cm}^{-1} \text{ mole}^{-1}$ at 600 m μ (Armstrong, 1964). The extinction coefficient for cytochrome *c* was taken as $18.5 \times 10^{-3} \text{ cm}^{-1} \text{ mole}^{-1}$ at 550 m μ (Margoliash, 1954).

Embryo age (days)	Glucose 6-phosphatase (μ moles of P _i liberated/20 min.)		ATPase (μ moles of P _i liberated/20 min.)		NADH ₂ -cytochrome <i>c</i> reductase (μ moles reduced/min.)		Diaphorase (μ mole reduced/min.)	
	(per g. of tissue)	(per mg. of protein)	(per g. of tissue)	(per mg. of protein)	(per g. of tissue)	(per mg. of protein)	(per g. of tissue)	(per mg. of protein)
6	8.3 $\pm$ 0.42 (6)	0.9 $\pm$ 0.07 (6)	40.0 $\pm$ 8.90 (6)	4.3 $\pm$ 1.23 (6)	1.1 $\pm$ 0.20 (4)	0.08 $\pm$ 0.01 (4)	0.2 $\pm$ 0.01 (4)	0.02 $\pm$ 0.001 (4)
9	17.6 $\pm$ 1.66 (5)	1.4 $\pm$ 0.14 (5)	54.6 $\pm$ 5.66 (5)	4.4 $\pm$ 0.66 (5)	2.0 $\pm$ 0.21 (6)	0.16 $\pm$ 0.018 (6)	0.50 $\pm$ 0.03 (6)	0.04 $\pm$ 0.002 (6)
12	20.6 $\pm$ 1.90 (6)	1.4 $\pm$ 0.16 (6)	56.4 $\pm$ 2.50 (6)	3.6 $\pm$ 4.20 (6)	2.2 $\pm$ 0.26 (5)	0.16 $\pm$ 0.027 (5)	0.57 $\pm$ 0.047 (6)	0.04 $\pm$ 0.003 (6)
16	29.8 $\pm$ 3.96 (7)	2.2 $\pm$ 0.34 (7)	93.7 $\pm$ 10.60 (7)	6.3 $\pm$ 0.71 (7)	2.7 $\pm$ 0.02 (7)	0.18 $\pm$ 0.028 (7)	0.9 $\pm$ 0.11 (7)	0.06 $\pm$ 0.009 (7)

some occasions succinate was added at the beginning of the enzyme assay with no resultant activity, though on the addition of NADH₂ reduction of cytochrome *c* or of the dye took place.

On examining the ultrastructure of the rough-vesicle microsomal subfraction (fraction II) it was found that at 6 days the bulk of fraction II consists almost entirely of osmiophilic granules, presumably ribosomes (Plate 2). Throughout development from 6 to 16 days, fraction II from embryonic chick livers contains progressively more rough vesicles (Plate 2). In the present study smooth membranes were already present in the two microsomal smooth-vesicle subfractions (Ia and Ib) at the earliest embryonic ages that were investigated (Plate 2). Fraction Ib always shows the presence of some free granules. Table 3 shows the changes in the distribution of proteins, phospholipid and RNA within fractions II, Ia and Ib as well as the combined smooth-membrane fraction Iab at various stages of development between 6 and 16 days.

From these results it becomes obvious that in all instances subfraction II, which consisted of free ribosomes and rough vesicles (whenever the latter were present), accounted for more than 70% of the protein, well over 60% of the phospholipid and (except at the 9-day stage, 77%) well over 80% of the RNA of the microsomal fraction (Table 3).

Subfraction II from 6-day-old embryonic chick liver consisted almost entirely of osmiophilic granules, yet, as pointed out above, at this stage there was already a great preponderance of protein over RNA and phospholipid (Table 3).

The two smooth-membrane fractions, which were separated by selective Mg^{2+} -binding (Dallner, 1963), varied quantitatively in relation to each other as development progressed (Table 3). Initially, at 6 days, subfraction Ib made up the bulk of the smooth-vesicle fraction, but with progressive development subfraction Ia increased and subfraction Ib decreased, until at 16 days no Mg^{2+} -binding vesicles (Ib) could be isolated.

The two types of smooth-vesicle fractions differ with respect to their RNA content, fraction Ib having a greater RNA concentration than fraction Ia. This may be correlated with the presence of clusters of osmiophilic granules in fraction Ib but not in fraction Ia (Plate 2). The interchange in the amount of the two smooth-membrane fractions is of some interest since there is now some evidence that the distribution of the phospholipid components making up the lipoprotein membranes of these two fractions is similar, whereas there are some important differences between the phospholipid composition of the two smooth-membrane fractions (Ia and Ib) and that of the rough-mem-

Table 3. *Protein, RNA and phospholipid concentrations within microsomal subfractions of different stages of embryonic chick liver*

Details of the preparations of the various microsomal subfractions and of the assay methods are presented in the Materials and Methods section. The results represent mean values $\pm$ S.E.M. The number of experiments from which the mean is derived is given in parenthesis. The results refer to values obtained for each fraction obtained from 1 g. of liver tissue. Fraction II, rough membranes; fractions Ia, Ib and Iab, smooth-membrane fractions. The livers were obtained from White Leghorn and White Leghorn $\times$ Australorp crossbred chick embryos.

Microsomal subfraction	Embryo age	Assays (mg./g. of tissue)			
		6 days	9 days	12 days	16 days
II	Protein	8.4 $\pm$ 0.16 (7)	11.9 $\pm$ 0.80 (14)	9.2 $\pm$ 0.53 (14)	9.6 $\pm$ 1.09 (9)
	Phospholipid	1.6 $\pm$ 0.09 (7)	2.5 $\pm$ 0.16 (13)	2.1 $\pm$ 0.07 (12)	3.0 $\pm$ 0.44 (9)
	RNA	2.7 $\pm$ 0.36 (5)	2.0 $\pm$ 0.08 (10)	2.4 $\pm$ 0.26 (9)	1.2 $\pm$ 0.14 (4)
Iab	Protein	2.4 $\pm$ 0.28 (3)	4.2 $\pm$ 0.58 (8)	3.7 $\pm$ 0.57 (6)	4.2 $\pm$ 0.38 (7)
	Phospholipid	0.5 $\pm$ 0.02 (2)	1.2 $\pm$ 0.16 (7)	0.9 $\pm$ 0.14 (6)	1.5 $\pm$ 0.22 (7)
	RNA	—	0.5 $\pm$ 0.11 (4)	0.2 $\pm$ 0.02 (2)	0.1 $\pm$ 0.02 (2)
Ia	Protein	0.3 $\pm$ 0.07 (4)	1.3 $\pm$ 0.21 (5)	1.2 $\pm$ 0.23 (3)	0
	Phospholipid	0.2 $\pm$ 0.08 (5)	0.4 $\pm$ 0.07 (4)	0.4 $\pm$ 0.16 (3)	0
	RNA	0.1 $\pm$ 0.02 (3)	0.1 $\pm$ 0.02 (5)	0.05 $\pm$ 0.017 (3)	0
Ib	Protein	1.3 $\pm$ 0.28 (3)	3.1 $\pm$ 0.71 (5)	1.8 $\pm$ 0.30 (3)	0
	Phospholipid	0.7 $\pm$ 0.06 (4)	0.9 $\pm$ 0.20 (4)	0.8 $\pm$ 0.27 (3)	0
	RNA	0.5 $\pm$ 0.17 (4)	0.5 $\pm$ 0.07 (5)	0.2 $\pm$ 0.08 (3)	0

Table 4. *Enzyme activities in microsomal subfractions of embryonic chick liver during development*

Cell fractionation techniques were as described in the Materials and Methods section. Fraction II, rough membranes; fraction Iab, combined smooth membranes. Assay conditions were as described in Table 2. The livers were obtained from White Leghorn $\times$ Australorp crossbred chick embryos. Incubation was for 20 min. at 30°.

Embryo age (days)	No. of experiments	Glucose 6-phosphatase				ATPase			
		μ moles of P _i /g. of tissue		μ moles of P _i /mg. of protein		μ moles of P _i /g. of tissue		μ moles of P _i /mg. of protein	
		II	Iab	II	Iab	II	Iab	II	Iab
6	3	19.1	6.4	2.3	2.5	30.2	25.4	3.7	11.0
9	4	59.0	8.0	4.8	2.4	34.8	41.2	3.2	11.7
12	3	41.4	9.6	4.7	2.3	18.6	41.1	2.0	8.5
16	5	55.4	11.2	5.2	2.7	45.7	58.3	3.5	11.3

brane fraction (II) (Ward, 1965), these differences being a higher sphingomyelin content and a concomitant lower phosphatidylcholine content in the smooth-membrane fractions (Ia and Ib) than that of the rough-membrane fraction (II). There is also a significant difference in the distribution of glucose 6-phosphatase and ATPase activities between the two smooth-membrane fractions (Ia and Ib) on the one hand and the rough-membrane fraction (II) on the other hand. Glucose 6-phosphatase activity was consistently and considerably greater in fraction II than in fraction Iab (Table 4). This distribution did not reflect only the fact that fraction II makes up over 70% of the microsomal protein, but with the exception of the 6-day stage the specific activities of fraction II for glucose

6-phosphatase were about twice as great as that for fraction Iab (Table 4). The ATPase activity of the microsomal fraction of the developing chick liver was fairly evenly distributed between the smooth- and the rough-membrane fractions (Table 4), as long as only the total activities were considered. The specific activities of all the developmental stages examined were, however, consistently greater in the smooth-membrane fractions than in the rough-membrane fractions (Table 4). The fact that in all instances the enzyme activities of the smooth-membrane fractions showed the same trend at 16 days, when only fraction Ib could be isolated, as at the other developmental stages, suggests that fractions Ia and Ib have a similar enzyme content at least as far as these two enzymes

are concerned. The differential distribution of these two enzymes implies that considerable differences exist between the smooth-membrane and the rough-membrane fractions and thus by analogy between the smooth and rough vesicles of the endoplasmic reticulum.

DISCUSSION

The amount of the membranous endoplasmic reticulum present in the liver cells of 6-day embryonic chicks is negligible (Karrer, 1960; Pollak, 1965; Pollak & Shorey, 1967). From 6 days onward the membrane content increases considerably with increasing developmental age, and there is a concomitant increase in the phospholipid content of the microsomal fraction. Between the developmental ages of 6 and 16 days the phospholipid content more than doubles (Table 1), whereas the protein content and the RNA concentration of the microsomal fractions stay relatively constant during this period (Table 1). At 6 days the hepatic microsomal fraction is almost entirely made up of osmiophilic and uranophilic granules (Plate 1), with negligible membranous endoplasmic reticulum, yet the protein/RNA ratio is well in excess of 3 (Table 1). In the 12-day embryonic liver, when the membranes of the endoplasmic reticulum are present to a significant extent, the protein/RNA ratio is 4, and at 16 days this ratio is about 5. This high protein/RNA ratio is to be expected when the microsomal fraction contains a large membranous component. The ribosomes, which are present in the microsomal fraction, are thought to consist of nearly equal amounts of protein and RNA (Hess & Lagg, 1963),

whereas the mean value of the protein/phospholipid ratio of liver microsomal membranes is about 2 (Ashworth & Green, 1966); hence one may calculate that in the microsomal fractions of embryonic chick livers up to the 12-day stage there is present a significant component consisting of one or more proteins that is of neither membrane nor ribosomal origin (Table 5). The isolation of this particulate protein microsomal subfraction was the topic of another communication (Pollak & Shorey, 1964b) that suggested that in embryonic chick liver Palade granules were either ribosomes or other osmiophilic granules (reticulosomes) made up of protein and phospholipid in the ratio of about 12.

By the sixteenth day of development these non-membranous non-ribosomal particulate proteins of chick liver (reticulosomes) reach negligibly low values (Table 5). The high values of these proteins during the early stages of development (Table 5), together with the low values at 16 days, when the liver cells were filled with membranes of the endoplasmic reticulum, led to the formation of the hypothesis that these proteins may be membrane precursors. On examining the analytical data of the protein, phospholipid and RNA values of the rough- (fraction II) and the smooth- (fractions Ia and Ib) microsomal fractions in conjunction with the electron micrographs of these fractions (Plate 2), it became even more apparent that, in spite of there being smooth membranes present in fraction Ia and Ib at the early stages of hepatic development, fraction II is practically free from membranes and exhibits very high protein/RNA and protein/phospholipid ratios, which decrease as morphogenesis progresses.

The actual decrease of microsomal RNA with

Table 5. *Amounts of non-membranous and non-ribosomal proteins of the hepatic microsomal fractions during development*

Column (2) shows the protein content of the microsomal fractions of embryonic chick liver (these values have been taken from Table 1). Column (3) represents membrane protein and is twice the phospholipid value as shown in Table 1, on the assumption that in microsomal membranes the protein/phospholipid ratio is 2 (Ashworth & Green, 1966). Column (4) represents ribosomal protein, which is taken to equal the RNA content of the microsomal fraction on the assumption that the ribosomal protein/RNA ratio is 1 (Hess & Lagg, 1963). Column (5) represents non-membranous and non-ribosomal protein and equals column (2) - [column (3) + column (4)].

(1) Embryo age (days)	(2) Microsomal protein (mg./g. of tissue)	(3) Membrane protein (mg./g. of tissue) (2 × phospholipid)	(4) Ribosomal protein (mg./g. of tissue) (1 × RNA)	(5) Non-membranous non-ribosomal protein
6	12.8	5.4	3.8	3.6
7	19.1	7.6	3.8	7.7
8	15.6	8.6	3.1	3.9
9	15.6	6.8	3.0	5.8
10	13.9	6.0	3.5	4.4
12	13.9	6.6	3.4	3.9
16	14.6	11.4	3.0	0.2

developmental age is difficult to interpret at this stage, but it confirms an earlier observation of a decrease of cytoplasmic RNA of embryonic chick liver during development (Duck-Chong *et al.* 1964).

Apart from a non-specific phosphatase (with β -glycerophosphate as a substrate) that was inactive at the pH (6.5) at which the enzyme assays of the microsomal fractions were carried out, all other enzymes assayed showed a steady increase between the developmental ages 6 and 16 days.

The results presented in Tables 2 and 4 show that from the early morphogenetic stages (6-day-old) the hepatic microsomes of the chick exhibit some glucose 6-phosphatase activity. In this respect the present results differ markedly from those obtained with guinea-pig and rat liver, in which glucose 6-phosphatase activity is absent during the early stages of development, and only develops towards the end of the gestation period and comes to a maximum shortly after parturition (Lea & Walker, 1964; Dallner *et al.* 1966a). On the other hand, human foetal liver possesses significant amounts of glucose 6-phosphatase relatively early in its development (Kilsheimer, Weber & Ashmore, 1960). Obviously there are considerable differences between species with respect to the timing of the appearance of glucose 6-phosphatase during morphogenesis. With the present state of knowledge on metabolic control mechanisms it is difficult to theorize about the significance of the appearance or absence of an enzyme such as glucose 6-phosphatase, since in this particular case most of the teleological discussions on the role of this enzyme in the developing embryo rest entirely on its action as phosphohydrolase in relation to gluconeogenesis. Nordlie & Arion (1964) showed in the first of a series of elegant investigations that the enzyme glucose 6-phosphatase has also pyrophosphatase and pyrophosphate-glucose-phosphotransferase activity. Thus in future discussions there can be no implicit assumption that, with respect to glycogen metabolism, hexokinase activity and glucose 6-phosphatase activity are opposed to one another, and substrate concentrations and the respective K_m values will have to be carefully considered before regarding this enzyme only as a phosphohydrolase.

It has been repeatedly demonstrated that glucose 6-phosphatase is a microsomal enzyme (for review see Dallner, 1963). In the present investigation glucose 6-phosphatase activity increased together with the formation of rough-endoplasmic reticulum (Table 4). By the ninth day of embryonic development there were already elements of rough endoplasmic reticulum present in embryonic chick liver, and the glucose 6-phosphatase activity was approximately twice as great as in 6-day embryonic chick liver. In embryonic rat liver, on the other

hand, the rough endoplasmic reticulum only commences to form between the fifteenth and eighteenth day of foetal development and by that stage there are no smooth vesicles present. By the time the rat is 1 day *post partum* the endoplasmic reticulum is completely developed (Moulé, 1964), and this is correlated with a dramatic rise in glucose 6-phosphatase activity (Dallner *et al.* 1966a).

In the chick there seems to be a correlation between the appearance of rough endoplasmic reticulum and glucose 6-phosphatase activity. The amount of glucose 6-phosphatase within the microsomal fraction of very early embryonic chick liver (6 days) is small but nevertheless real, and may possibly be explained in terms of the presence of reticulosomes possessing glucose 6-phosphatase activity. Reticulosomes exhibit a high specific activity of glucose 6-phosphatase (J. K. Pollak, unpublished work).

The results in Table 4 give some information on the localization of glucose 6-phosphatase and ATPase within the microsomal subfractions. Thus glucose 6-phosphatase activity is predominantly associated with the rough-microsomal fraction (fraction II), whereas ATPase tends to fractionate equally with the smooth- (fractions Ia and Ib) and the rough- (fraction II) microsomal fractions (Table 4). However, the specific activities of ATPase/mg. of protein were considerably higher in the smooth-membrane fractions of the hepatic microsomes than in the rough membranes (Table 4). This differential enzyme distribution between the smooth- and the rough-membrane fractions suggests that in developing chick liver the rough and the smooth elements of the endoplasmic reticulum cannot be regarded as interconvertible, in the sense that the smooth endoplasmic reticulum is derived from the rough by the removal of ribosomes as has been suggested for embryonic rat liver by Dallner *et al.* (1966a,b).

Recent work with embryonic chick liver, showing that the phospholipid compositions of fraction Iab and fraction II differ from each other (Ward, 1965), further supports the concept that in the chick at any rate the rough- and the smooth-microsomal fractions are chemically and enzymically different.

The authors are indebted to Miss Maria Woog for her excellent technical assistance with certain phases of the work, and to Mr C. D. Shorey of the Electron Microscope Unit of the University of Sydney for the electron micrographs. This work was supported by grants of the New South Wales State Cancer Council and the Australian Research Grants Committee. The authors are also indebted to the National Health and Medical Research Council and the University of Sydney Cancer Research Fund for a grant to purchase the Beckman-Spinco model L2 centrifuge used for the latter part of the work.

REFERENCES

- Allen, R. J. L. (1940). *Biochem. J.* **34**, 858.
- Armstrong, J. McD. (1964). *Biochim. biophys. Acta*, **86**, 194.
- Ashworth, L. A. E. & Green, C. (1966). *Science*, **151**, 210.
- Caquoin-Carnot, M. & Roux, J. M. (1960). *Bull. Soc. Chim. biol., Paris*, **42**, 237.
- Chen, P. S., Toribara, T. Y. & Warner, H. (1956). *Analyt. Chem.* **28**, 1756.
- Dallner, G. (1963). *Acta path. microbiol. scand.* (Suppl.), p. 166.
- Dallner, G., Siekevitz, P. & Palade, G. E. (1966a). *J. Cell Biol.* **30**, 73.
- Dallner, G., Siekevitz, P. & Palade, G. E. (1966b). *J. Cell Biol.*, **30**, 97.
- Duck-Chong, C., Pollak, J. K. & North, R. J. (1964). *J. Cell Biol.* **20**, 25.
- Ernster, L., Siekevitz, P. & Palade, G. E. (1962). *J. Cell Biol.* **15**, 541.
- Hess, E. L. & Lagg, S. E. (1963). *Biochemistry*, **2**, 726.
- Karrer, H. E. (1960). *J. Ultrastruct. Res.* **4**, 149.
- Keilin, D. & Hartree, E. F. (1945). *Biochem. J.* **39**, 289.
- Kilsheimer, G. S., Weber, D. R. & Ashmore, J. (1960). *Proc. Soc. exp. Biol., N.Y.*, **104**, 515.
- Lea, M. A. & Walker, D. G. (1964). *Biochem. J.* **91**, 418.
- Lillie, F. R. (1952). *Development of the Chick*, p. 70. Revised by Hamilton, H. L. New York: Hewey Holt and Co.
- Margoliash, E. (1954). *Biochem. J.* **56**, 535.
- Moulé, Y. (1964). In *Cellular Membranes in Development*, p. 124. Ed. by Locke, N. New York: Academic Press Inc.
- Nordlie, R. C. & Arion, W. J. (1964). *J. biol. Chem.* **239**, 1680.
- Pollak, J. K. (1965). *Abstr. Aust. biochem. Soc.* p. 48.
- Pollak, J. K. & Shorey, C. D. (1964a). *Abstr. 6th int. Congr. Biochem., New York*, VIII-82.
- Pollak, J. K. & Shorey, C. D. (1964b). *Biochem. J.* **93**, 36c.
- Pollack, J. K. & Shorey, C. D. (1967). *Aust. J. exp. Biol. med. Sci.* (in the Press).
- Rothschild, J. (1963). *Symp. biochem. Soc.* **22**, 4.
- Siekevitz, P. (1963). *Annu. Rev. Physiol.* **25**, 15.
- Swanson, M. A. (1955). In *Methods of Enzymology*, vol. 2, p. 541. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Ward, D. (1965). M.Sc. Thesis: University of Sydney.