STUDIES ON THE BIOCHEMISTRY OF MITOCHONDRIA AND CELL MORPHOLOGY IN THE NEONATAL SWINE HEPATOCYTE

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ABSTRACT

Mitochondrial preparations isolated from neonatal swine hepatocytes show a marked increase in oxidative and concomitant phosphorylative capacity between birth and 2 days postpartum. There are no changes in the coupling parameters (respiratory control ratio and adenosine diphosphate/O ratio) with age. Changes in sedimentation properties in a sucrose gradient suggest qualitative changes in the mitochondria. Some of the lipid measurements (increased phospholipid) might be interpreted as supportive of this suggestion, although most could also be regarded as indicative of quantitative changes (increased number of mitochondria). Electron microscopy of isolated mitochondria and of the hepatocyte demonstrated an increased number of mitochondria but no change in shape, size, or structure as the pig developed. An increase in a number of cytoplasmic components (Golgi apparatus and endoplasmic reticulum) and a decrease in glycogen were also observed. The functional changes in mitochondria seem to occur within a short period of time (6–12 hr postpartum).

The newborn piglet is metabolically immature and generally subject to a number of environmental stresses during its first hours of life. A number of these metabolic immaturities persist for several days after birth and may contribute to the high mortality of piglets which approaches 30% in many swine-rearing operations (1). Initially, the piglet has large stores of glycogen in both muscle and liver (2, 3), a somewhat low blood glucose and a high blood fructose (which it cannot use and excretes within 24 hr) (4, 5), very small amounts of depot fat (less than 2%) (6), and a limited capacity to mobilize fatty acids when exposed to starvation (7–9). In addition, the newborn piglet is extremely sensitive to cold stress (10) and has a marginal capacity to carry on gluconeogenesis at birth (3, 8, 9, 11).

In order to discern the extent of change occurring in mitochondrial function as the newborn piglet adapts to an aerobic atmosphere and as part of an ongoing program to study a number of aspects of carbohydrate metabolism and hepatocyte structure in neonatal pigs, we have examined a number of aspects of hepatocyte mitochondrial structure and function in neonatal pigs.

METHODS

Pregnant sows of mixed genetic origin (primarily Duroc × Chester White crosses) were obtained from a local breeder and farrowed at the Shell Development Company facilities in Modesto, Calif.[1]

[1] The research described in the report involved animals maintained in animal care facilities fully accredited by the American Association for Accreditation of Laboratory Animal Care.

Two piglets were removed from the sow immediately after birth, before suckling, and were designated day 0 animals. On the 2nd (40–60 hr), 6th (5–7 days), and 14th (13–15 days) days, two pigs were removed from the sow 2 hr before sacrifice. The animals selected were a male and a female if the litter size and sex distribution allowed. The 65-day-old pigs were obtained from a local breeder. For the study of the changes occurring between day 0 and day 2, two pigs were removed from one sow at 0, 6, 12, 24, and 48 hr postfarrowing.

Piglets were sacrificed by cervical dislocation and exsanguination, and the 65-day pigs were sacrificed with a captive-bolt gun. A portion of the liver was frozen in liquid nitrogen and stored in a freezer at −20°C for glycogen determination (12). Another portion of the liver was chilled in 0.25 M sucrose—0.05 M Tris, pH 7.4. A liver homogenate was made (1 g + 9 ml isolation medium) with a motor-driven Teflon-glass homogenizer. The isolation medium was 2 mM *N*-2-hydroxyethylpiperazine-*N*¹-2-ethane sulfonic acid (HEPES), 220 mM mannitol, 70 mM sucrose, 0.1 mM ethylenediaminetetraacetate (EDTA) pH 7.4 at 4°C (adjusted with KOH). A mitochondrial fraction was isolated essentially as previously indicated (13), using 800 *g* × 10 min for the low-speed centrifugations and 16,000 *g* × 15 min for the high-speed centrifugations (20,000 *g* × 15 min for day 0 preparations). The high-speed supernates were removed with a pipette in day 0 preparations since the mitochondria did not form a firm pellet. The final pellet (from 1 g liver) was suspended in 1.0 ml of isolation medium.

Oxidase activity and concomitant phosphorylation were determined with a Gilson Electronics Oxygraph (Gilson Medical Electronics Inc., Middleton, Wis.) with a Clark electrode (Clark Equipment Company, Buchanan, Mich.) at 30°C. The incubation medium (final concentration) was 220 mM mannitol, 10 mM glycylglycine, 10 mM KPO_4, 2.5 mM $MgCl_2$, 0.5 mM EDTA, and contained 0.9 mg/ml of defatted bovine serum albumin (15). The pH was adjusted to 7.4 with KOH. The final volume of the incubation was 2.0 ml. The addition order was the incubation medium premix (1.5 ml) and H_2O, then 0.2 ml of mitochondrial suspension yielding state I respiration, then substrate yielding state IV respiration, and finally 400 nmoles of adenosine diphosphate (ADP) yielding state III respiration followed by state IV respiration when the ADP was exhausted. The final substrate concentrations were succinate or glutamate or α-ketoglutarate—2.5 mM, DL-β-hydroxybutarate—5.0 mM, or 2.5 mM pyruvate and 0.25 mM malate.

Protein was determined by the method of Gornall et al. (16).

Lipids were extracted from 1.0 ml of the mitochondrial suspension according to the method of Folch et al. (17), using $CHCl_3$:CH_3OH (2:1). The washed extracts were dried with air, resuspended in $CHCl_3$, and portions were used to determine total lipids gravimetrically and phospholipid phosphorus by the Bartlett procedure (18).

Gradient centrifugation was conducted with a Sorvall HB-4-swinging-bucket head (Ivan Sorvall, Inc., Norwalk, Conn.) with linear sucrose gradients (made with a Beckman gradient maker). The gradients used were 25–55% sucrose in 20 mM KPO_4, pH 7.4. Centrifugation was carried out for 2 hr at 20,000 *g*. The tubes were analyzed by scanning at 280 nm with an ISCO gradient scanning device (Instrumentation Specialties Co., Lincoln, Neb.). The gradient was removed from the top of the tube by pumping 65% sucrose into the bottom of the tube.

For the electron microscopy, small pieces of liver were immediately removed from the animal, cut into 1 mm cubes, and fixed in 1% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.3. The samples were postfixed in buffered 1% osmium tetroxide, dehydrated in alcohol and propylene oxide, and embedded in an Epon-Araldite mixture. Sections were stained with uranyl acetate and lead citrate. To examine isolated mitochondria, a sample of the normally prepared, resuspended mitochondrial fraction was recentrifuged in isolation medium; the supernate was decanted and replaced by glutaraldehyde fixative. After 15 min, the pellet was loosened and transferred to fresh fixative and treated in the same manner as a tissue block.

RESULTS

Oxidative Activity

The oxidative activity and concomitant phosphorylative capacity of isolated mitochondrial fractions with various substrates are indicated in Table I. The rate of oxygen uptake with all substrates was markedly enhanced between day 0 and day 2. The increased activity was indicated by state I (endogenous substrates), state IV (added substrate), and state III (added substrate and ADP) respiration rates. The increase was true for all substrates studied, although the magnitude of this increase varied among substrates (from three- to fivefold). Although the state IV rates are not tabulated, quantitative changes similar to the state III rates occurred as indicated by the relatively constant respiratory control ratios. After the very large increase between day 0 and day 2, the rates generally declined to a lesser value at day 14, which seemed to continue to day 65. The chronological pattern is somewhat different for different substrates.

TABLE I
Mitochondrial Oxidase Activity

Substrate	Day 0	Day 2	Day 6	Day 14	Day 65
A. Succinate					
SA_p*	29.3 ± 4.10	103.0 ± 5.90	102.3 ± 5.26	67.3 ± 4.90	60.0 ± 9.25
RC‡	5.7 ± 0.36	6.3 ± 0.51	5.9 ± 0.24	6.0 ± 0.40	7.1 ± 0.52
ADP/O§	1.61 ± 0.022	1.72 ± 0.064	1.63 ± 0.045	1.71 ± 0.094	1.62 ± 0.065
n‖	13/14	14/14	14/14	10/10	5/5
B. Pyruvate + malate					
SA_p	18.8 ± 2.50	50.4 ± 3.37	39.2 ± 2.97	25.8 ± 2.58	26.6 ± 3.67
RC	5.9 ± 0.55	6.9 ± 0.69	4.5 ± 0.26	4.5 ± 0.28	6.0 ± 1.06
ADP/O	2.36 ± 0.060	2.44 ± 0.056	2.38 ± 0.052	2.35 ± 0.038	2.35 ± 0.053
n	12/14	14/14	14/14	10/10	5/5
C. Glutamate					
SA_p	21.3 ± 2.87	69.1 ± 5.38	59.9 ± 4.10	29.7 ± 2.74	22.8 ± 3.26
RC	6.4 ± 0.70	9.6 ± 0.96	6.4 ± 0.45	5.7 ± 0.32	5.3 ± 1.13
ADP/O	2.31 ± 0.031	2.36 ± 0.057	2.39 ± 0.045	2.39 ± 0.052	2.36 ± 0.072
n	10/14	14/14	13/14	10/10	5/5
D. α-Ketoglutarate					
SA_p	9.6 ± 1.81	46.2 ± 4.07	33.0 ± 2.71	21.4 ± 2.36	18.3 ± 3.60
RC	3.5 ± 0.32	7.7 ± 1.63	4.0 ± 0.31	4.4 ± 0.36	3.7 ± 0.54
ADP/O	2.31 ± 0.325	2.35 ± 0.058	2.20 ± 0.062	2.20 ± 0.044	2.21 ± 0.067
n	11/14	14/14	14/14	10/10	5/5
E. β-Hydroxy-butyrate					
SA_p	5.0 ± 0.43	23.3 ± 2.14	17.2 ± 1.05	12.5 ± 1.20	10.7 ± 1.81
RC	3.3 ± 0.76	4.7 ± 0.58	2.9 ± 0.20	3.4 ± 0.36	2.9 ± 0.23
ADP/O	2.52 ± 0.326	2.10 ± 0.087	1.99 ± 0.061	2.01 ± 0.072	2.05 ± 0.110
n	9/12	14/14	14/14	10/10	5/5
F. None					
RR I¶	2.5 ± 0.25	5.3 ± 0.24	4.6 ± 0.24	3.3 ± 0.29	3.2 ± 0.40
	13/14	14/14	14/14	10/10	5/5

* State III respiration rates expressed as natoms O/min per mg protein (mean ± SEM).
‡ Respiratory control ratio = rate of state III respiration per rate of state IV respiration (mean ± SEM).
§ nmoles ADP utilized/min per mg protein:natoms 0 (state III)/min per mg protein (mean ± SEM).
‖ Number of animals per group showing respiratory control per total number of animals assayed. Averages based on those showing RC only.
¶ State I respiration rates expressed as natoms 0/min per mg protein (mean ± SEM).

The phosphorylative rates (for all substrates) changed in parallel with the state III respiration rates so that the ADP/O ratios remained constant at all ages (Table I). The ADP/O ratio is a direct measure of the coupling of oxidation of substrate with phosphorylation, and the experimentally determined ratios indicate reasonable coupling for these preparations, except with α-ketoglutarate which has a theoretical ADP/O ratio of 4. The low ADP/O ratios achieved with this substrate may indicate a defect in the substrate level phosphorylation accompanying the metabolism of this compound. There were no striking changes in respiratory control ratios (an indirect measure of the coupling of oxidation to phosphorylation) with age, using a number of substrates. There may have been transitory increases in the ratios on day 2 with several substrates, but the variability was greater and the significance not apparent.

It should be noted that a number of preparations

TABLE II
State III Respiration Changes

Substrate	Day	SA_p*	%	SA_w‡	%
A. Succinate	0	29.3	100	685	100
	2	103.0	350	1512	221
	6	102.3	348	1704	249
	14	67.3	230	1910	279
	65	60.0	205	1374	195
B. Pyruvate + malate	0	18.8	100	440	100
	2	50.4	268	741	168
	6	39.2	208	655	149
	14	25.8	137	733	167
	65	26.6	142	610	139
C. Glutamate	0	21.3	100	498	100
	2	69.1	320	1003	201
	6	49.9	234	834	168
	14	29.7	140	845	170
	65	22.8	107	522	105
D. α-Ketoglutarate	0	9.6	100	224	100
	2	46.2	462	680	304
	6	33.0	344	551	246
	14	21.4	233	608	271
	65	18.3	191	419	187
E. β-Hydroxybutyrate	0	5.0	100	117	100
	2	23.3	465	343	293
	6	17.2	344	288	246
	14	12.5	250	355	303
	65	10.7	214	246	210

* SA_p = state III respiration, natoms oxygen/min per mg protein.
‡ SA_w = state III respiration, natoms oxygen/min per g liver.

did not show good respiratory control, i.e., either they did not return to state IV after state III (preparations did not shut down) or, when ADP was added during state IV, there was no increase of oxygen uptake characteristic of state III. In either case, the preparations were considered defective and further studies are not included because the results were meaningless. The defective preparations were almost exclusively from day 0 animals.

An expression of state III respiration calculated on both a protein and wet-weight basis is indicated in Table II. Compared to the expression on a protein basis, the wet-weight expression shows the amount of increase generally to be less, and the temporal pattern sometimes different. On a wet-weight basis, there was increased activity by day 2 which remained elevated for a longer period. The expression of rates on a protein basis assumes that the majority of the protein in the isolated fraction was of mitochondrial origin. This is a false assumption, particularly in the day 0 preparations which were severely contaminated by endoplasmic reticulum, as indicated by electron microscopy. The calculation on a wet-weight basis assumes that the yield of mitochondria from a fixed weight of tissue (2 g) at each age was constant, so that the increase with age in the oxidative rates probably represents a larger number of mitochondria isolated per gram of liver in the older pigs. Since the mitochondrial preparations were contaminated by extraneous protein, we feel that the wet-weight expression of oxidative rates more closely represents the in vivo state.

The day 0 mitochondrial fraction was different from that of other ages in that it did not form a pellet when spun at 20,000 $g \times 15$ min in either the first or second high-speed centrifugation. It formed a loosely packed fraction at the bottom of the tube from which the supernate could not be decanted but was pipetted off. Since the first high-speed supernate contained much of the glycogen from the cell (up to 240 mg/g liver in the newborn animals as compared to 50–75 mg/g liver in older animals), this could hinder sedimentation of

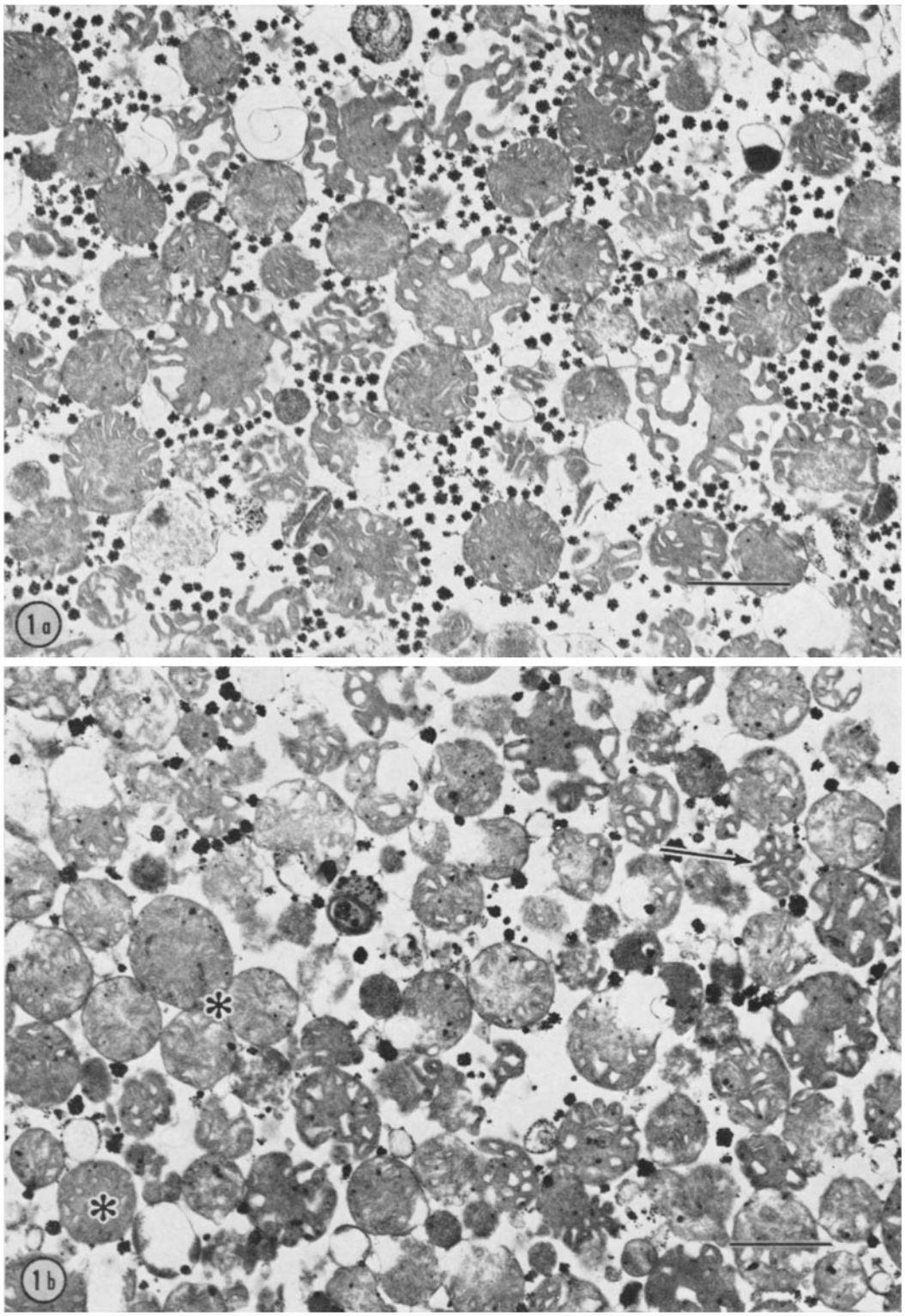

FIGURE 1 Electron micrographs of isolated mitochondrial pellets. (*a*) Mitochondria from a day 0 animal. There is a large amount of glycogen present, and the condensed mitochondrial configuration is predominant. (*b*) Mitochondria from a day 6 animal. Glycogen is present and the mitochondria are in both the orthodox (asterisks) and condensed (arrow) configurations. The scale bar indicates 1 μ. × 15,900.

TABLE III

Development of Oxidase Activity—Day 0 to Day 2

Substrate		Time (hr)*				
		0	6	12	24	48
A. Succinate	SA_w‡	278	277	1660	1410	1770
	RC§	6.0	2.8	3.7	4.1	4.3
	ADP/O‖	1.54	1.97	1.46	1.39	1.56
B. Pyruvate + malate	SA_w	106	121	665	609	735
	RC	2.3	1.6	3.4	5.8	4.2
	ADP/O	2.73	3.41¶	2.17	2.20	2.39
C. Glutamate	SA_w	159	189	1118	950	976
	RC	5.8	2.5	4.6	6.0	5.3
	ADP/O	2.17	1.93	2.28	2.09	2.35
D. α-Ketoglutarate	SA_w	63	60	427	358	435
	RC	3.5	1.3	2.4	2.5	2.7
	ADP/O	3.07	2.96	2.06	2.08	2.14
E. β-Hydroxybutyrate	SA_w	40	42	196	173	262
	RC	2.0	2.3	2.9	2.6	2.9
	ADP/O	7.06¶	6.21¶	1.39	1.55	1.50

* Data are the average of two animals at each time.

‡ SA_w = state III respiration, natoms oxygen/min per g liver.

§ Respiratory control ratio = rate of state III respiration per rate of state IV respiration.

‖ nmoles ADP utilized/min per g liver:natoms O (state III)/min per g liver.

¶ These excessive ADP/O ratios are due to lack of good respiratory control in these preparations with the particular substrate.

the mitochondria. However, this supernate was discarded and replaced by isolation medium for the second high-speed centrifugation so that the failure to sediment was probably due to a change in density, in shape, or in size of the mitochondrial particles and not to increased viscosity of the medium caused by the excessive glycogen present. In contrast, the preparations from day 2 animals formed a pellet which was even more tightly packed in the day 6 and older preparations.

Since there was no apparent solution to the pellet problem in day 0 preparations, none of the preparations from the various age groups were washed; as a result, most mitochondrial fractions were severely contaminated by broken mitochondria and endoplasmic reticulum as indicated by electron micrographs. In addition, the fractions usually contained rather large amounts of glycogen, since the animals were all fed. There was generally a sharp line of demarcation between the mitochondrial portion of the pellet and the contaminating material, although glycogen was distributed throughout. The preparations from older animals had less contamination by endoplasmic reticulum, and the amount of glycogen was variable. There seemed to be less glycogen in preparations from day 2 animals, and this correlates with chemical evaluations of hepatic glycogen which tend to be minimal on day 2 (3, 32). The mitochondrial fraction was examined from three different day 0 pigs and, in all cases, the majority of mitochondria were in the condensed form (terminology of Hackenbrock [19]), with very few particles exhibiting the orthodox configuration (Fig. 1 *a*). The mitochondria varied greatly in size but exhibited no particular size distribution from the top to the bottom of the sedimented pellet. A number of swollen mitochondria appeared throughout. In older piglets (day 2, 6, or 14), there were generally more mitochondria in the orthodox configuration than on day 0 (Fig. 1 *b*), although the proportion in orthodox configuration (in these older animals) varied with the individual animal and did not seem to be correlated with the

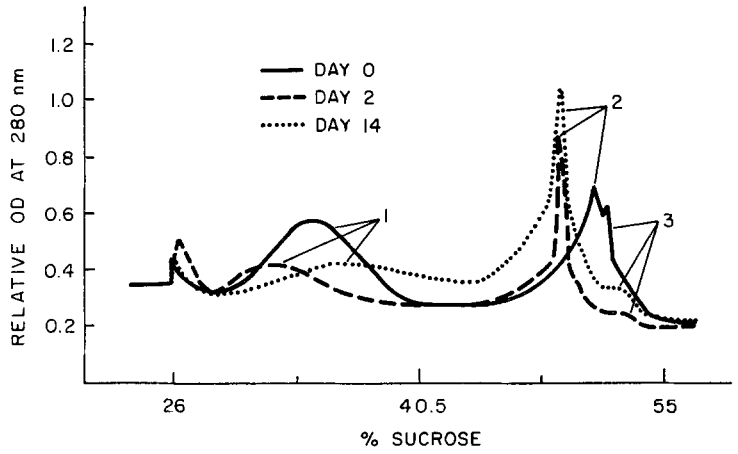

FIGURE 2 Sedimentation of mitochondrial fractions through a sucrose gradient. A liver mitochondrial sample (0.2 ml) was placed on top of 12.0 ml of a sucrose gradient 25–55% and centrifuged at 20,000 *g* for 2 hr. The traces are representative of those examined. Preparations from 4 day 0, 5 day 2, 5 day 7, and 6 day 14 animals were examined. Fractions 1, 2, and 3 as discussed in the text are indicated.

piglet's age. There was no apparent change in size or shape of mitochondria or in number or arrangement of cristae with age. In preparations from older animals, several types of extraneous particles were present with either single or double membranes. The nature of these particles was not apparent.

In order to determine more precisely at which time the large changes in mitochondrial activity occurred, piglets obtained from one sow were sacrificed at 0, 6, 12, 24, and 48 hr after birth (all within 1 hr of the time indicated) and mitochondrial activity was studied. The results presented in Table III indicate that the changes in activity occurred between 6 and 12 hr postpartum and that the change was for all substrates. It seems that whatever the mechanism of increased activity, it is affecting a large number of mitochondrial activities during a short time span. The mechanism of this increase in activity was not apparent, although it seemed probable that whole units of mitochondrial activity were increasing more or less at once, i.e., the capacity to oxidize all substrates examined increased at the same time. This might be the result of synthesis of new mitochondria or of some type of activation. Although the increase in activity was rather abrupt and affected all substrates, the maintenance of the enhanced activity was different for each enzyme, possibly indicating differential rates of turnover.

Composition of Mitochondria

Samples of the isolated mitochondrial fraction were centrifuged in a sucrose gradient to determine if the change in differential centrifugation properties could be explained by a difference in density of the particles. Gradient fractions 2 (fraction sedimenting at about 47–51% sucrose) and 3 (fraction sedimenting at about 51–53% sucrose) from day 0 piglets sedimented to a greater extent than comparable fractions from day 2 and older piglets (Fig. 2), although the mitochondrial fractions from older pigs sedimented more readily by differential centrifugation than those from day 0 animals. Succinoxidase activity was recovered in fractions 2 and 3 from the gradient, while fraction 1 (fraction sedimenting at about 31–36%) contained predominantly glycogen and no succinoxidase. Although there were variations in the absolute patterns seen from animal to animal on a particular day, the patterns in Fig. 2 are representative of those usually seen. Material absorbing at 280 nm was more equally distributed between fraction 2 and 3 in day 0 samples compared to day 2 and day 14 where fraction 2 was the major fraction. There were also shifts in the amount and density of fraction 1 on the various days. It is not apparent whether the two more evenly distributed fractions (2 and 3) on day 0 represent two populations of mitochondria.

From day 0 to day 2, there was a marked increase in total lipid and phospholipid content in

TABLE IV
Lipid Analysis of Mitochondrial Preparations

Day	mg total lipid / mg protein	mg phospholipid / mg protein	Phospholipid (% of total)	mg total lipid / ml mitochondria	mg phospholipid / ml mitochondria	Phospholipid (% of total)
0	0.342 ± 0.062	0.088 ± 0.016	25.7	7.73 ± 1.47	1.95 ± 0.202	25.2
2	0.640 ± 0.045	0.207 ± 0.012	33.8	9.40 ± 0.89	2.88 ± 0.141	30.7
6	0.503 ± 0.059	0.187 ± 0.014	37.2	8.29 ± 1.29	2.93 ± 0.185	35.4
14	0.283 ± 0.062	0.101 ± 0.019	35.7	5.54 ± 0.41	2.60 ± 0.180	47.0

the mitochondrial fraction when the data are expressed on a protein basis (Table IV). Part of this increase is explicable because of the large amount of protein in the fraction on day 0 which decreased by day 2. Much of this protein was due to the excessive amounts of particulate glycogen and microsomal contamination observed with day 0 preparations. This complication was at least partially obviated when the lipid values were expressed per milliliter of isolated mitochondrial fraction. In this case, the change in total lipid was much less, while the change in phospholipid was still rather marked. Presumably, the phospholipid was present in mitochondrial membranes and the microsomes which were contaminants.

Hepatocyte Ultrastructure (Figs. 3 and 4)

CELL MEMBRANE AND NUCLEUS: There were no major changes in the structure of the cell membrane with age. The tight junctions, bile canalicules with projecting microvilli, as well as microvillar projections into the space of Disse, were all well developed at birth. The nucleus occupied a peripheral position in the newborn, while at day 2 (and thereafter), a more central position was observed. The nuclear membrane with abundant nuclear pores, a well-separated inner and outer membrane (contiguous with the rough endoplasmic reticulum), as well as chromatin and a prominent nucleolus, were evident from birth.

GOLGI APPARATUS AND ENDOPLASMIC RETICULUM: There was an increase between birth and day 2 in the amount of Golgi apparatus and smooth endoplasmic reticulum present, as well as a relocation from a peripheral to a more random orientation within the cell. In addition, the Golgi apparatus had more expanded membranes and partially filled saccules on day 2 and at older ages (as opposed to flat, empty membranes on day 0). From a peripheral and perinuclear location in the newborn, the endoplasmic reticulum proliferated extensively to occupy a nearly universal location throughout the cell in day 2 and older animals. In addition, the cisternae were generally more distended at the older ages.

MICROBODIES, LYSOSOMES, AND INCLUSION BODIES: On the day of birth, there were structures present which appeared to be bounded by a double membrane and contained a moderately dense, amorphous matrix. In older animals (>day 2) there were, in addition, a large number of particles bounded by a single membrane. The identification of these types of particles remains open, although they might be related to microbodies and/or to lysosomes as suggested by Bischoff et al. (22). Ellipsoid inclusions (unknown function) as previously described by Bischoff et al. (22) were seen on the day of birth, with few by day 2 and none observed at later ages. In some cells on day 0 and day 2, there were numerous grouped lipid droplets which did not occupy any particular location in the cells, while in piglets 6 and 14 days of age there was little evidence of lipid deposition in the liver.

GLYCOGEN: The most prominent entity seen in electron micrographs of the newborn piglet hepatocyte was particulate glycogen. It might be estimated that in most cells well over one-half of the volume was occupied by glycogen, which existed as individual rosettes somewhat intermediate between the α and β particles described in rat hepatocytes by Dallner et al. (20). By day 2, the amount of glycogen was reduced to minimal amounts and existed mostly as scattered individual particles throughout the cytoplasm. In 6- and 14-day piglets, there was more glycogen than at day 2, and it was generally distributed in patches.

MITOCHONDRIA: In the newborn, the hepatic mitochondria were located peripherally in the cell. Most were spherical in shape, but there were also elongated forms and some branched forms. Cristae were present and the mitochondria looked

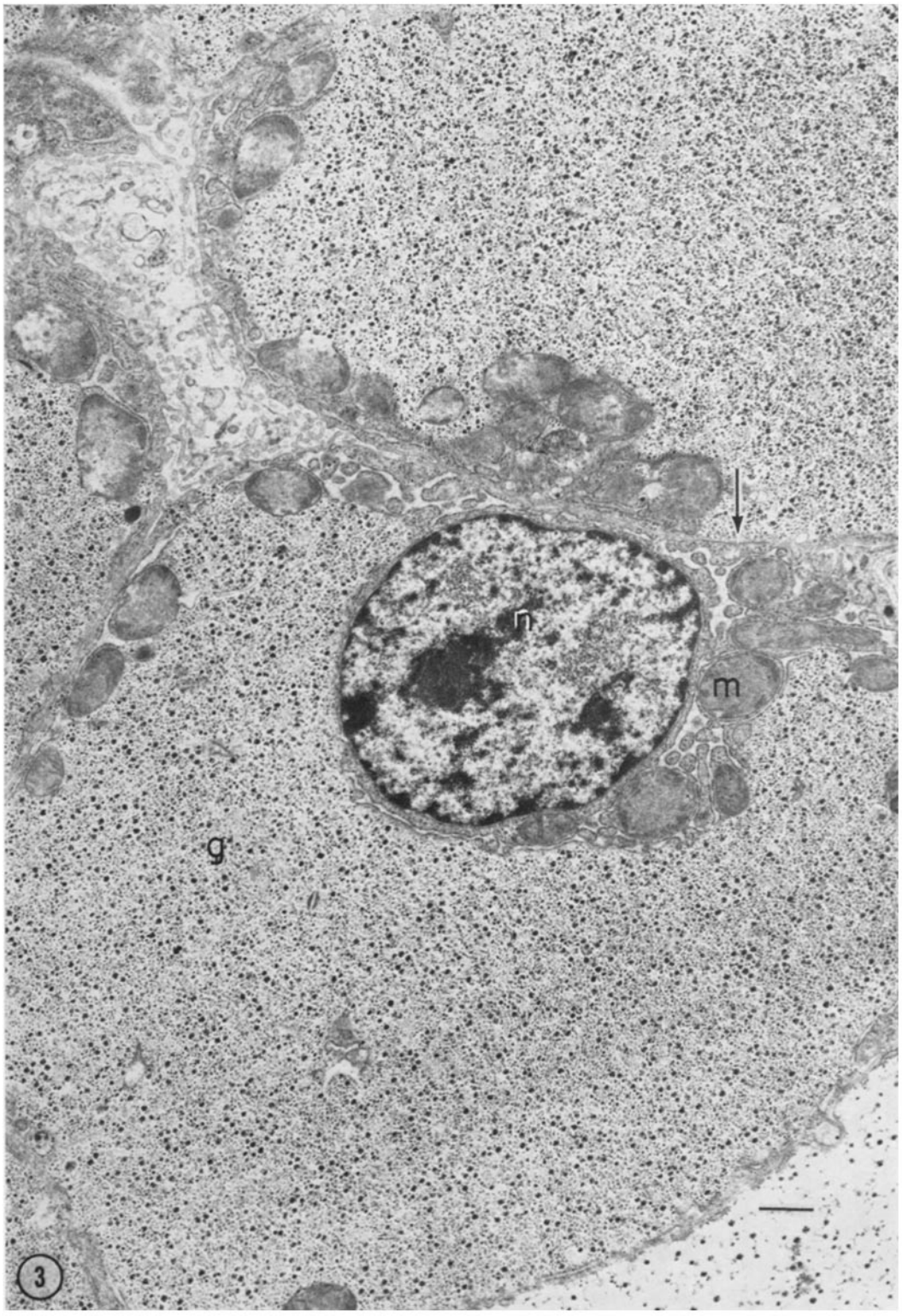

FIGURE 3 Hepatocytes from day 0 piglet. Symbols indicate nucleus (*n*), mitochondria (*m*), glycogen (*g*), terminal bars (arrow). The scale bar indicates 1 μ. × 8800.

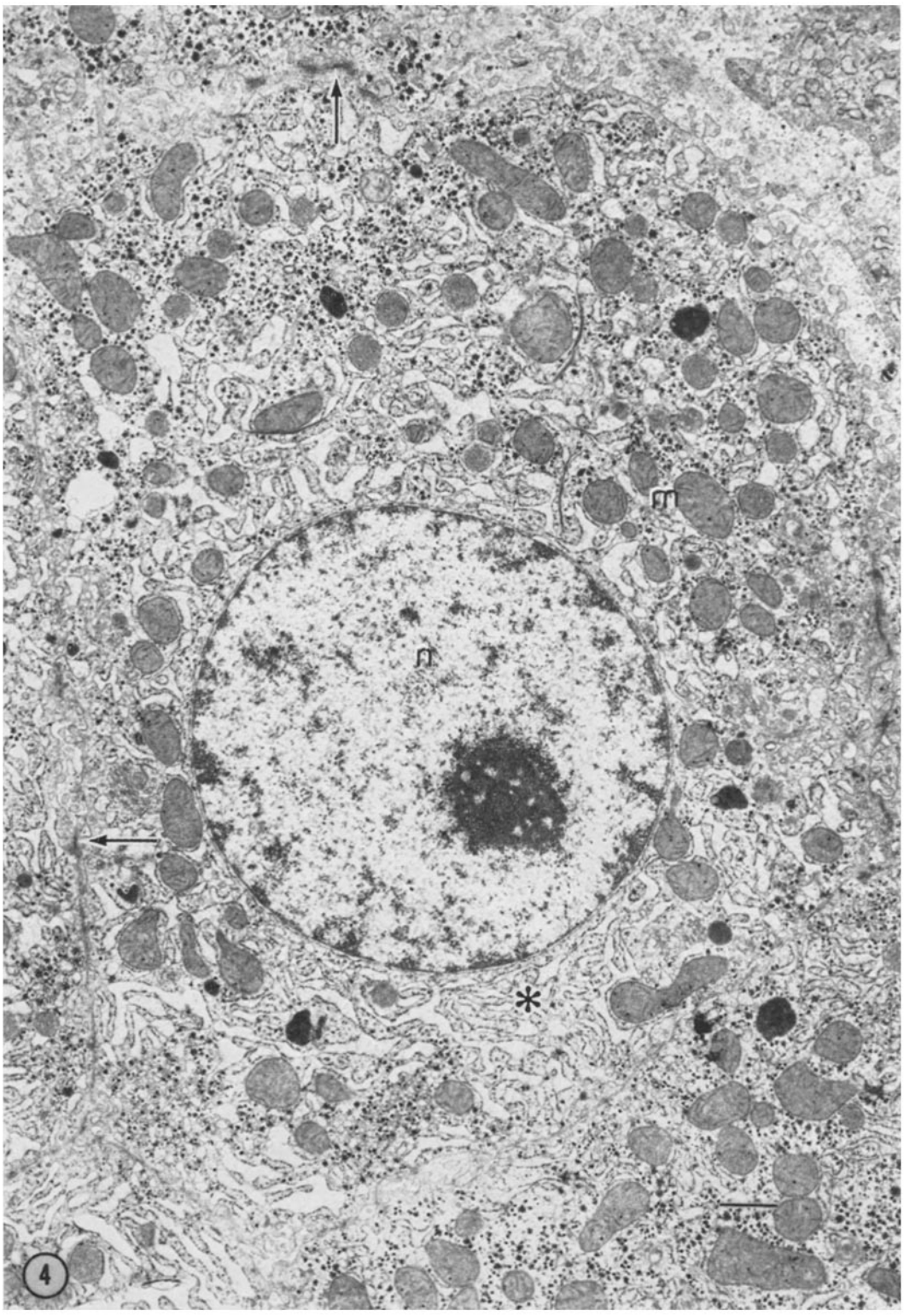

FIGURE 4 Hepatocyte from day 14 piglet. Symbols indicate nucleus (*n*), mitochondria (*m*), terminal bars (arrows), and rough endoplasmic reticulum (asterisk). Note the glycogen present in isolated patches. The scale bar indicates 1 μ. × 8800.

much like typical hepatocyte mitochondria. By day 2, there was a large increase in the number of these organelles per cell, and the location was now more universal in the cytoplasm. Although we believe that there was no particular change in the size, shape, structure, or amount of cristae but only in number of mitochondria per cell with age, many mitochondria appeared swollen in sections from day 0 animals. Since the isolated preparations of mitochondria from day 0 animals did not demonstrate this tendency to swell, the problem may have been one of fixative penetration. (A number of fixatives and conditions were used to attempt to obviate this problem, as well as the washing out of the excessive glycogen depots, but none improved the fixation.) The tendency toward swelling in *in situ* mitochondria might also reflect changes in the particles themselves, as indicated by the changes in centrifugal behavior already discussed.

DISCUSSION

It is apparent that there are large changes in the oxidative activity of swine hepatocyte mitochondria between the time of birth and about 2 days postpartum (Tables I–III). That there are essentially no changes in the coupling parameters (respiratory control [RC] ratio and ADP/O ratio) suggests that the mitochondria are changing primarily in a quantitative rather than qualitative manner, i.e., that there are more mitochondria or more functional mitochondrial subunits.

Comparable changes in neonatal liver from other species have been demonstrated. The number of mitochondria per gram wet-weight and the mitochondrial diameter increased as the rat developed from neonate to adult, while the succinate dehydrogenase did not seem to change (25). The increase in mitochondrial number had been previously demonstrated (29), while a recent study confirmed these results (34) and calculated the volume fraction occupied by the mitochondria in neonatal rat liver. A study of mouse liver has shown a decrease in hematopoietic cells and a concomitant increase in hepatocytes from birth to 30 days (21). A light- and electron microscope study of the developing swine hepatocyte (22) has shown a twofold increase in the cell size after birth. The cytoplasmic:nuclear ratio increased after birth in mouse hepatocytes (33). Most of these studies indicate, either directly or indirectly, an increase in mitochondrial number in the developing mammalian hepatocyte.

Indications of an increase in functional units of mitochondria are an increase in oxygen uptake and pyruvate utilization in rabbit liver slices between birth and 4 days (24) and a doubling of oxygen uptake in rat liver slices between late fetal and early postnatal life (26). The pyridine nucleotide content (30), the enzymes involved in flavine coenzyme production (31), and the cytochrome content (23) all increased after birth in rats. The cytochrome increase occurred before birth in guinea pigs, which are generally considered to be metabolically more mature at birth. A reduced yield of mitochondria from fetal rat liver (compared to adults), as well as a lack of respiratory control, was shown with rat hepatocyte (27, 37) and bovine heart (28) preparations. Krebs' cycle enzymes have been studied in neonatal rat liver. Succinate dehydrogenase increased just before birth (35), while malate and α-ketoglutarate dehydrogenases and citrate synthase did not change with age (35–37). Aconitase, fumarase, and β-hydroxybutyrate dehydrogenase increased rather markedly immediately before and after birth. The time course of development was specific for each enzyme, and apparently short-term synthesis of intact mitochondria did not occur in developing hepatocytes of this species as opposed to our observations on swine hepatocytes.

The increase in oxidative activity and the increase in phospholipid content (suggesting increased membrane content) between day 0 and day 2 are compatible with the observations by electron microscopy of an increased number of mitochondria per cell. Although opposite in nature and thus not interpretable at this time, the changes in centrifugal properties, by both gradient and differential centrifugation, indicate qualitative changes in the mitochondria. Electron microscopy, on the other hand, indicated no change in size, shape, or number of cristae of mitochondria, either in isolated preparations or *in situ*. (The tendency for *in situ* mitochondria to appear swollen may reflect fixative penetration as previously mentioned or possibly a qualitative change in membrane properties.) Recently, studies by Pollak and Munn (38) have demonstrated a decrease in density (as estimated by gradient centrifugation) in postnatal rat liver mitochondria which they attributed to development of the impermeability of the inner mitochondrial membrane to sucrose. Hallman and Kankare (39) also have shown a decrease in density of mitochondria from perinatal

rat liver and suggest that this represents a maturation of the inner mitochondrial membrane. These density changes are similar to our findings with swine hepatocyte mitochondria.

In summary, we cannot yet detail the changes seen in the developing swine hepatocyte, but the evidence points to increased number of mitochondria per cell and to some lesser qualitative changes in the mitochondria. This synthesis of mitochondria seems to occur in a very short time span (6–12 hr postpartum), which suggests many fascinating questions for future pursuit.

The gross structural changes observed in the postnatal swine hepatocyte (increased Golgi apparatus and smooth endoplasmic reticulum, great proliferation of rough endoplasmic reticulum and mitochondria, and large decrease in particulate glycogen) have been reported by Bischoff et al. (22), and our observations are in general agreement. We have observed neither a close proximity of the Golgi apparatus to the bile canalicules in day 0 hepatocytes, as indicated by Bischoff et al. (22), nor an association of the smooth endoplasmic reticulum with glycogen patches in older animals as indicated by Dallner et al. (20) in rat hepatocytes. Except for these minor points, the major observation to be emphasized by our study is the increase in mitochondrial number. The large increase in cellular synthetic activity occurring after birth has been demonstrated by Dallner et al. (20) in rat hepatocytes.

The observation by Bischoff et al. (22) that there is an increase in cell size (light microscopy) greatly magnifies the large increases in amount and number of subcellular organelles which occur between day 0 and day 2. Although Bischoff et al. presented a micrograph of a hepatocyte 21 hr after birth which had greatly proliferated organelles, we do not know if these changes occur gradually over this time span or abruptly (between 6 and 12 hr postpartum) as do the mitochondrial changes.

Received for publication 19 July 1971, and in revised form 24 January 1972.

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