A Cytochemical Study of Lipid, Glycogen and Lactate Dehydrogenase in the Developing Liver[1]

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In recent studies the lactate dehydrogenase (LDH) isozymes of adult (Lindsay, '63) and embryonic (Philip and Vesell, '62; Nebel and Conklin, '64) chick liver were demonstrated. A maximum of six isozymes were found in the embryonic liver while four isozymes were present in the liver after hatching. While several theories (Apella and Markert, '61; Markert, '62; Cahn et al., '62; Fritz and Jacobson, '63) have been proposed to account for the number of isozymes, there have been few studies which have attempted to correlate LDH isozymes with either intracellular distribution (Allen, '61) or metabolic function (Lindsay, '63; Plagemann et al., '60).

The fact that the major changes in the LDH isozymes were noted during the period from the sixth to the twelfth day of development, the time of onset of carbohydrate and lipid metabolism in hepatic cells of the chick (Dalton, '37), suggested a possible correlation between these events. Furthermore, the isozyme changes might be associated not only with the onset of specific function but also with changes in the intracellular distribution of lactate dehydrogenase. In the present study the intracellular deposition of glycogen and lipid and the localization of lactate dehydrogenase activity were demonstrated in order to determine whether any of these factors could be correlated with the isozyme pattern previously reported (Nebel and Conklin, '64). In addition, the application of histochemical methods for revealing LDH activity afforded the opportunity to evaluate certain current problems of histochemical technique, i.e., the use of formalin-fixed (Walker and Seligman, '63) vs. acetone extracted (Hitzeman, '63) vs. fresh tissue for the demonstration of oxidative enzyme activity. Finally, in spite of increasing evidence to the contrary (Schneider and Hogeboom, '56; Thorne, '60; Conklin et al., '62; Strittmatter, '63) there are continued reports (Walker, '63; Walker and Seligman, '63) of the exclusive intramitochondrial localization of all oxidative enzymes. Therefore, an attempt was made to corroborate previous findings (Conklin et al., '62) on the intracellular localization of lactate dehydrogenase.

MATERIALS AND METHODS

The material utilized in this study consisted of samples of liver from either six, 12 or 18 day white leghorn chick embryos or five day chicks (5 days post-hatch). The embryos were staged according to the Hamburger-Hamilton ('51) system and the ages reported were derived from this classification.

Most excised liver samples were prepared for sectioning by placing the tissue on dry ice until frozen solid. The frozen sample was then mounted on a microtome chuck and sectioned in a cryostat at −20°C. Initially, tissue samples were frozen by quenching in either liquid nitrogen, or in isopentane cooled to −150°C with liquid nitrogen, but this method was abandoned because of the excessive tissue damage (ice crystal artifact) which occurred. Frozen sections were mounted on a cover slip and either (1) fixed for five minutes in cold (4°C) 10% neutral buffered formalin or 10% neutral formalin containing 2% calcium chloride (frozen-fixed sections), (2) dipped in cold (4°C) acetone (extracted sections) for 15 seconds (Hitzeman, '63), or (3) utilized directly (fresh sections). In addition, a few tissue samples were cut into approximately 1 mm cubes and fixed for 10–15 minutes

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in the cold formalin solutions prior to freezing and cryostat sectioning (fixed-frozen). The sections obtained from these samples were utilized without additional treatment. Glycogen was demonstrated in fixed-frozen and frozen-fixed sections by means of the periodic acid Schiff (PAS) technique (Mowry, '58) controlled with amylase digestion. Lipid was demonstrated in either fresh, fixed-frozen or frozen-fixed sections by staining with 0.7% Sudan black B in propylene glycol. Lactate dehydrogenase activity was revealed by incubating fresh, fixed-frozen, frozen-fixed and acetone extracted sections in a substrate containing sodium lactate (0.1 M); oxidized nicotinamide adeninedinucleotide (NAD) (0.3 mg/ml); phenazine methosulfate (PMS) (1.0 μg/ml); Nitro BT (0.5 mg/ml) and tris hydroxyaminomethylmethane (Tris) buffer (0.20 M) for 15 minutes. The final pH of the solution was 7.0. Mitochondria were demonstrated by fixing samples of liver tissue in Regauds' fluid followed by post-chromation, double embedding in celloidin and paraffin, and staining with a modification (Sevringhaus and Thompson, '39) of Altmann's acid fuchsin procedure.

After the PAS reaction or staining with aniline acid fuchsin, the tissue sections were dehydrated in graded alcohols, cleared in xylene and mounted in a synthetic resin (HSR). After either lipid staining or the demonstration of LDH activity tissue sections were rinsed in distilled water and mounted in glycerogel.

OBSERVATIONS

Observations of glycogen and lipid inclusions were based on the study of either fixed-frozen or frozen-fixed tissues. This was permissible since there were no differences in the results after the two methods of preparation. Unless otherwise noted, the descriptions of the distribution of lactate dehydrogenase activity were from the study of acetone extracted tissue sections. After other methods of tissue preparation, which preserved the lipid of the hepatic cells, i.e., fixed-frozen, frozen-fixed and fresh sections, obvious artifactual distribution of formazan occurred. The intensity of the reaction in acetone extracted sections was as great as the reaction in fresh untreated sections while formalin-fixed sections exhibited a marked reduction in mitochondrial enzyme activity. In those stages in which a cytoplasmic LDH was present, the activity of the cytoplasmic enzyme was completely inhibited in fixed-frozen and frozen-fixed tissue sections. More detailed descriptions of the several staining procedures are present below.

Six day liver

The hepatic cells were approximately hexagonal in shape, contained small nuclei, and were arranged in loose cords of cells which were separated by irregular vascular spaces. The coarse granular cytoplasm of the hepatic cells was lightly PAS positive (fig. 5) and also stained with Sudan black B (fig. 1). However, the staining was homogenous and occurred throughout the cytoplasm and it was not possible to see whether the substances stained were small lipid and glycogen inclusions or the ground cytoplasm. After amylase digestion there appeared to be a slight decrease in the intensity of the PAS reaction indicating the presence of a small amount of glycogen. There appeared to be no regional (intralobular) differences in the distribution of either glycogen or lipid. Lactate dehydrogenase activity (fig. 9) was localized in small granules which were distributed throughout the cytoplasm but were most numerous immediately adjacent to the nuclear membrane. The size and shape of these granules suggested that all LDH activity was localized within the mitochondria since there was no background activity within the cytoplasm. An attempt was made to demonstrate mitochondrial morphology by fixation and staining but all attempts to demonstrate the organelle in 6 and 12 day livers were unsuccessful. Acid fuchsin staining occurred throughout the cytoplasm and dedifferentiation of the stain could not be obtained by the usual technique. Mitochondria could be demonstrated in hepatic cells of 18 day embryonic and five day chick liver without any difficulty. Regauds' fixative appeared to give better preservation of structure and greater facilitation of staining of embryonic mitochondria than did Zenker formol.

Twelve day liver

At 12 days the hepatic cell cords were aligned more closely together resulting in smaller intercellular channels and the organization of definitive sinusoids. The pattern of the veins could not be assessed and there was no evidence of the organization of hepatic lobules. The hepatic cells had increased in size (fig. 10) and the nuclei contained prominent nucleoli.

After Sudan black staining, the cytoplasm of the hepatic cells was only lightly colored and numerous, small, lipid droplets were present in the cytoplasm (fig. 2). While conclusive evidence was not obtained, the ease of extraction of much of the lipid in cold acetone suggested that the largest part of a lipid droplet was neutral fat.

The cytoplasm of the hepatic cells was still PAS positive but, in addition, small angular inclusions were present (fig. 6). These were glycogen inclusions as indicated by their digestibility in alpha amylase. Again, no regional distribution of either lipid or glycogen was noted. In the 12 day liver (fig. 10) formazan was distributed both in a granular and a diffuse form throughout the cytoplasm of the hepatic cells indicating a dual localization of lactate dehydrogenase activity. In spite of acetone extraction, a small amount of lipid artifact was present (fig. 10). However, as noted previously, the extent of lipid artifact was much less after acetone extraction of fresh sections than it was when either fresh, fixed-frozen or frozen-fixed sections (fig. 12) were employed.

Eighteen day liver

The hepatic cells were arranged in cords of cells with many venous channels interspersed between them. While there was still no organization of definitive lobules a regional distribution of glycogen was evident with the heaviest concentration occurring adjacent to the venous channels. The glycogen was present in much greater quantity than in earlier stages (fig. 7). The quantity of lipid had also increased (fig. 3). The distribution of lipid was as previously noted with no evidence of regional variation. The ground cytoplasm of the hepatic cells no longer exhibited an affinity for either PAS or Sudan black. The distribution of lactate dehydrogenase activity (fig. 11) was similar to that observed in the 12 day liver with activity being both granular and diffuse in nature. There was an apparent increase in activity as indicated by the greater deposition of formazan in the hepatic cells of the 18 day liver. Figure 13 illustrates LDH activity in a fresh, nonextracted section of 18 day liver and clearly demonstrates the gross artifact which occurred when large quantities of lipid were present. Staining of the mitochondria with aniline acid fuchsin revealed them to be rod-like and closely associated with the lipid vacuoles or surrounding the nuclear membrane (not pictured, but similar to fig. 15). Comparison of the distribution of LDH activity with mitochondrial distribution clearly revealed that LDH activity was localized both intra- and extramitochondrially.

Five day chick liver

The cytoplasm of liver cells of the five day chick contained numerous lipid droplets (figs. 4 and 18). A few of these droplets were as large as or exceeded the diameter of the nucleus while others were just large enough to be discernible. Glycogen inclusions were present in the cytoplasm between the lipid droplets (figs. 8 and 18). Less glycogen was present than was noted in the 18 day embryonic liver. There was no apparent regional distribution of either glycogen or lipid in the five day chick liver. Lactate dehydrogenase activity was confined to filamentous structures (fig. 14) which lay in close proximity to the lipid vacuoles and nuclei within the hepatic cells. Comparison of the LDH activity with the morphology and distribution of mitochondria (fig. 15) demonstrated that all LDH activity was confined to the mitochondria. When nonextracted fresh sections were employed for the demonstration of LDH activity (fig. 16) most of the formazan was present in the many lipid droplets with little or no indication of activity in the mitochondria.

DISCUSSION

As noted by Dalton ('37) and confirmed in the present study a small amount of glycogen was present in the cells of the six day liver. The entire cytoplasm of the

hepatic cells was homogenously PAS positive (fig. 5) and distinct granular inclusions were not discernible. After amylase digestion the intensity of the PAS reaction was reduced indicating that some of the reactive material was glycogen. Distinct glycogen inclusions were first demonstrated in the 12 day liver (fig. 6), appeared to reach a peak concentration at 18 days (fig. 7) and declined slightly after hatching (fig. 8). Similar changes were noted by Ballard and Oliver ('63) who found the glycogen content of the ten day liver to be very low (7 mg/gm) with a four fold increase occurring by the nineteenth day (26 mg/gm). The peak of glyconeogenesis occurred about the fifteenth day of development. The increase in glycogen content was paralleled by an increase in glycogen synthesizing enzymes.

In the six day liver the cytoplasm of the hepatic cells demonstrated an affinity for Sudan black B not unlike that seen after the PAS reaction. The material which stained with Sudan black was lipid in nature but it was not determined whether stored lipid or cytoplasmic structural lipid was responsible for the staining. While glycogen deposition reached a peak during the embryonic period, lipid deposition differed in that from the first appearance of lipid inclusions at 12 days (fig. 2) there was a further increase at the subsequent stages examined (figs. 3 and 4). While there were slightly heavier accumulations of glycogen along the venous channels, a regional distribution of lipid was not evident. This is in contrast to the intralobular pattern of liver glycogen and lipid described in other species (Novikoff and Essner, '60).

The numerous lipid droplets in the hepatic cells seriously interfered with precise enzyme localization when cryostat sections of either fresh, fixed-frozen, or frozen-fixed tissues were employed (figs. 12, 13 and 16) since these methods of preparation preserved the lipid inclusions. Examination of the slides revealed that the lipid droplets contained much of the formazan while there was little or no staining of the mitochondria. While it was not possible to conclusively determine if the tetrazolium salt was initially and selectively bound to the lipid inclusions, it seemed doubtful since immediately following the reaction only a small amount of formazan was absorbed to the surface of the droplets. However, in time, the formazan was observed to increase at the interfaces of the lipid droplets and eventually seemed to fill an entire droplet. The accumulation of formazan in the lipid was accompanied by "destaining" of the mitochondria. This change in the distribution of the formazan makes it rather unlikely that lipid artifact is due to electrostatic binding between the formazan and lipoprotein (Pearse and Hess, '61; Liu and Baker, '63), especially since other intracellular lipoprotein membranes did not exhibit a similar affinity for the formazan. The suggestion of Novikoff et al. ('61) that the causative factor is preferential solubility at a lipid-aqueous interface seems most plausible.

The problem of lipid interference was easily overcome by employing short extraction in cold acetone (Hitzeman, '63). Not only did this procedure remove the majority of the interfering lipids (figs. 9, 10, 11 and 14) but there appeared to be little loss of enzyme activity. This was in contrast to the results obtained after formalin fixation (fixed-frozen and frozen-fixed). Although several investigators (Walker and Seligman, '63; Liu and Baker, '63) favor the utilization of fixed sections for enzyme localization it should be realized that formalin fixation results in protein (enzyme) denaturation (Baker, '58), thus reducing total enzyme activity. This denaturation may even be selective (figs. 10 and 12) thus giving rise to false negative results.

A final requisite to accurate enzyme localization is the use of a simple yet effective substrate which promotes a rapid reaction rate. With the substrate employed, electrons are rapidly transferred from the reduced NAD via phenazine methosulfate to the tetrazolium salt (Dewey and Conklin, '60; Conklin et al., '62). This simple mechanism provided for precise enzyme localization since (1) the reaction rate was very rapid thus precluding diffusion of either enzyme or end product in the absence of interfering factors and (2) the tetrazolium was reduced as the direct result of dehydrogenase activity and not secondarily by means of a diaphorase.

Lactate dehydrogenase activity was observed to vary in localization during development. At the onset (6 day) all LDH activity was confined to the mitochondria (fig. 9). At 12 (fig. 10) and 18 days (fig. 11), the liver LDH activity was localized both intra- and extramitochondrially. After hatching, all LDH activity was again confined to the mitochondria (fig. 14). In order to confirm the localization of LDH activity, the distribution and morphology of the mitochondria were demonstrated by staining with aniline acid fuchsin (fig. 15). While this method was ineffective, for some unknown reason, on 6 and 12 day liver, the mitochondria were easily demonstrated in the hepatic cells of 18 day and five day post-hatch liver and clearly confirmed the dual localization of LDH activity in the embryonic hepatic cells (figs. 11 and 15). The close proximity of the mitochondria to the lipid droplets, previously noted by Rosa and Tsou ('63), probably contributed to the diffusion of the formazan when the lipid was not extracted.

In a previous report (Conklin et al., '62), the intracellular localization of oxidative enzymes was demonstrated in both frozen and living fibroblasts by cytochemical procedures. In that study it was quite apparent that certain enzymes were confined to the mitochondria, certain enzymes were extramitochondrial in localization and certain enzymes exhibited a dual localization. Lactate dehydrogenase was one of the latter enzymes in that it was located both intra- and extramitochondrially. In contrast to this finding is the report of Walker ('63) that lactate dehydrogenase has an exclusively mitochondrial localization. His report is in contradiction not only to the observations in this study but to biochemical evidence (Murthy and Rappoport, '63) as well. The discrepancy between Walker's results and others may be due to the utilization of formalin fixed tissues (Walker and Seligman, '63; Walker, '63) for enzyme studies. As demonstrated in the present study, formaldehyde appeared to selectively inhibit non-mitochondrial dehydrogenase activity.

Solomon ('59) in a biochemical study of developing chick liver reported that all LDH activity was localized in the supernatant and none in the mitochondria during all stages of development. Solomon's findings are subject to criticism on two points: (1) the assumption that there was no change in mitochondrial size during development and (2) the assumption that there was no difference in the solubility of the enzymes studied. As illustrated in this study, the mitochondria of the six day liver are small ovoid bodies (fig. 9) while by the end of the developmental period they are long filamentous structures (figs. 14 and 15). This change in size is undoubtedly accompanied by changes in mass which alter the sedimentation properties of the mitochondria. In addition, as Zeigler and Linnane ('58) have illustrated, mitochondrial LDH is a very soluble enzyme thus requiring very gentle handling in order to demonstrate its mitochondrial distribution. A final argument for the localization of LDH as demonstrated in the present study is that at different stages, under similar conditions of procedure, the enzyme was observed to vary in localization. It is of interest that Solomon ('59) found the greatest total LDH activity to occur between the eighth and twentieth day. In the present study this is the period during which LDH was observed to have a dual localization.

Previously, the LDH isozymes of developing chick liver were described (Nebel and Conklin, '64). It was reported that the six day liver contained two isozymes, LDH-3 and -4. Liver from seven to ten day embryos contained four isozymes, LDH-1, -2, -3 and -4, while from the twelfth to the twenty-first day, five isozymes, LDH-1, -2, -3, -4, and -5 were present. After hatching isozyme LDH-5 was lost and only four isozymes were found, i.e., LDH -1, -2, -3 and -4. Only general correlations can be made between the isozyme pattern and lipid, glycogen and enzyme content. The deposition of glycogen and lipid began at 10–12 days at about the time when the full complement of LDH isozymes appeared. While lipid deposition continued, glycogen deposition decreased, coincident with the loss of isozyme LDH-5. The presence of LDH-5 was also coincident with the presence of extramitochondrial LDH activity. In fact, there was an obvious increase in extramitochondrial LDH (figs.

10 and 11) as demonstrated cytochemically during the same period that LDH-5 was present (Nebel and Conklin, '64) and at the same time that a three-fold increase in supernatant LDH occurred (Solomon, '59).

If isozyme LDH-5 is indeed an extramitochondrial LDH, then isozymes LDH-1, -2, -3 and -4 are probably located in the mitochondria. Of these, LDH -3 and -4 seem to be basic to the mitochondria since they were the only isozymes present in the early stages of development when all the LDH was localized in the mitochondria (fig. 9). With the onset of more complex metabolic function at days ten to twelve, isozymes LDH -1 and -2 were added to the mitochondrial complement of isozymes. This change in LDH isozymes probably marked the shift from an anaerobic to a chiefly aerobic metabolism within the hepatic cells. As has been demonstrated (Lindsay, '63) the presence of the LDH-1 type of isozymes is characteristic of organs with a high oxidative metabolism.

While Solomon ('59) has discussed the concept that mitochondrial enzymes are synthesized in the "supernatant," the fact that all activity was confined to the mitochondria in the early stages of development suggests that this organelle may be the source of these enzymes. However, since the impermeability of the intact mitochondrial membrane (Zeigler and Linnane, '58) makes it unlikely that transmembrane diffusion of large protein molecules occurs, it seems more plausible that intra- and extramitochondrial proteins are synthesized *in situ*. This does not obviate the possibility that specific LDH subunits are synthesized at different intracellular sites. In fact, this is an appealing concept in view of the isozyme changes which occur during development (Nebel and Conklin, '64).

As described earlier, isozyme LDH-5 may be an extramitochondrial enzyme. It should be noted, however, that under certain conditions other LDH isozymes may also be localized extramitochondrially. When liver from a ten day embryo was transplanted to the chorioallantoic membrane of a ten day host and cultured for seven days a marked increase in LDH activity occurred (fig. 17). The distribution of the LDH was the same as observed in the 18 day embryonic liver, i.e., both intra- and extramitochondrial. However, when the LDH isozymes of the liver transplant were demonstrated (Nebel and Conklin, '64) it was found that only isozymes LDH-2, -3 and -4 were present. While the significance of this finding remains to be elucidated, it does demonstrate that both enzyme content and distribution are subject to variation. The nature and extent of this variation is currently under study.

SUMMARY

1. Glycogen and lipid content and lactate dehydrogenase (LDH) activity were demonstrated in chick liver by cytochemical procedures.

2. Prominent glycogen inclusions first appeared on the twelfth day of development, became most prominent by the eighteenth day, and declined slightly after hatching.

3. Lipid deposition progressively increased throughout the period studied. Marked accumulation of lipid did not occur until after the twelfth day of development.

4. At six days, all LDH activity was localized within the mitochondria. At 12 and 18 days, the enzyme was localized both intra- and extramitochondrially while after hatching all activity was again present only within the mitochondria.

5. The distribution of LDH activity and glycogen and lipid content was somewhat correlative with the isozyme content of the hepatic cells.

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PLATE 1

EXPLANATION OF FIGURES

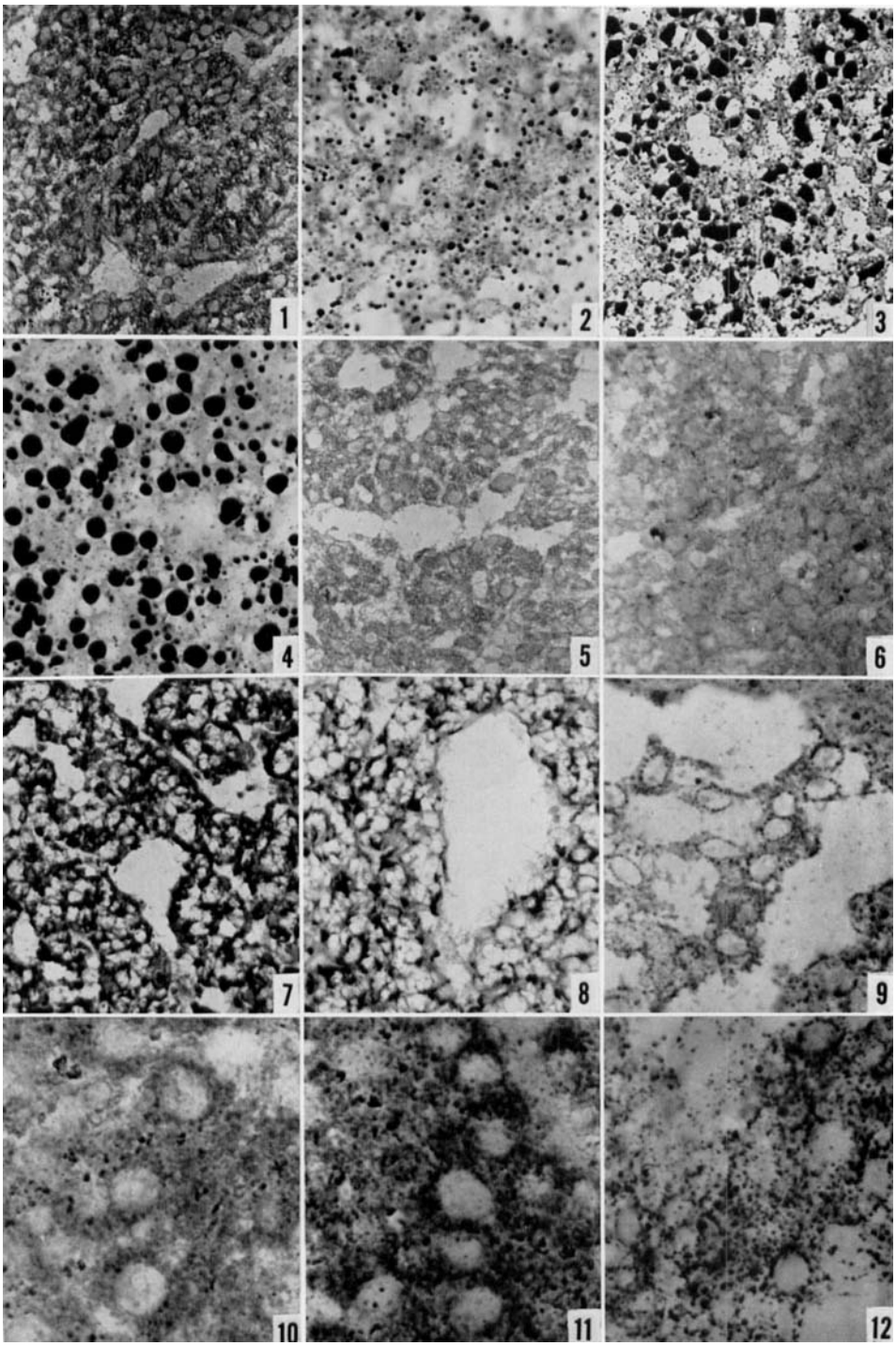
All figures are of chick liver

1–4 Liver from six, 12 and 18 day embryos and five day chick, respectively, demonstrating intracellular lipid. Note the decreasing affinity of the cytoplasm for the stain with the progressive accumulation of large lipid droplets. Contrast with figure 18. Sudan black B. × 700.

5–8 Liver from six, 12 and 18 day embryos and five day chick, respectively, demonstrating intracellular glycogen. At six days only the cytoplasm is slightly PAS positive while by 12 days a few small glycogen inclusions are present. At 18 days a large amount of glycogen is present which decreases after hatching. Contrast with figure 18. PAS. × 700.

9–11 Liver from six, 12 and 18 day embryos, respectively, demonstrating lactate dehydrogenase activity after acetone extraction of lipids. Note the localization of activity in the mitochondria of six day hepatic cells while activity is present in both the mitochondria and cytoplasm of the 12 and 18 day liver cells. Also note the gradual increase in activity with increasing age. A small amount of artifactual localization may be observed in figures 10 and 11 due to the incomplete extraction of lipid. × 1,700.

12 Lactate dehydrogenase activity in a 12 day liver after formalin fixation. Note the loss of all extramitochondrial activity. Observe also that there is marked artifactual localization of activity in the preserved lipid droplets (see figs. 2 and 10). × 1,700.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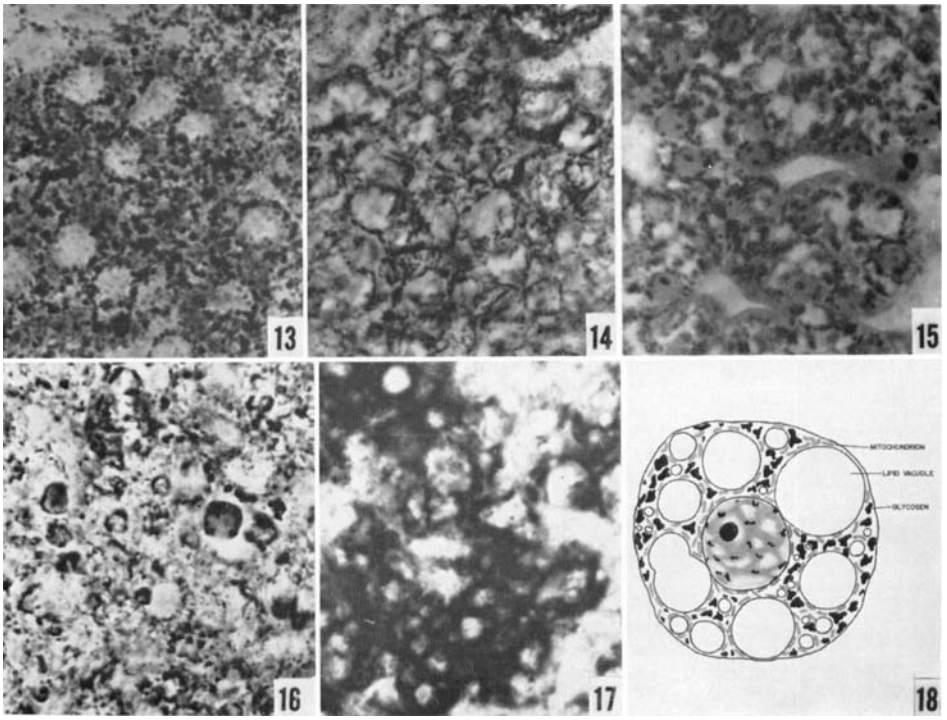

13 Lactate dehydrogenase activity in a non-extracted 18 day liver. There is gross artifactual absorption of the formazan to the lipid droplets (see figs. 3 and 11). × 1,700.

14 Lactate dehydrogenase activity in an acetone extracted section of five day chick liver. The activity is confined to the filamentous mitochondria which lie in close proximity to the lipid droplets (see figs. 4 and 18). × 1,700.

15 The demonstration of mitochondria after Regaud's fixation and staining with aniline acid fuchsin. The distribution of the numerous rod-like mitochondria is similar to that demonstrated in figure 14. × 1,700.

16 Lactate dehydrogenase activity in a non-extracted five day chick liver. The pronounced absorption of the formazan to the lipid droplets completely prevents the accurate localization of enzyme activity. × 1,700.

17 Lactate dehydrogenase activity in an acetone extracted ten day liver which had been transplanted to the chick chorioallantoic membrane for seven days (total age = 17 days). Localization of activity appears to be both intra- and extramitochondrial and of much greater intensity than in non-transplanted liver of comparable age (fig. 11). × 1,700.

18 A composite diagram of a liver cell from a five day chick illustrating the intracellular distribution of lipid, glycogen and mitochondria.