the contribution of the pentose pathway(1) through heptose and shikimic acid(12); on the other hand, leucine (and eventually valine) should be synthesized from acetate-1-C^{14}.

Formate is extensively used by *Past. multocida* for synthesis of amino acids (Table VI) and purine bases(13). The relatively higher incorporation of C^{14} into the serine group (glycine-serine-cystine-cysteic acid) is consistent with formate fixation in serine as described in animal tissues(14). After transformation into pyruvic acid (serine dehydrase reaction), serine can label the Krebs cycle intermediates and related amino acids. However, the possibility of direct incorporation of formate into pyruvate(15) cannot be ruled out. The synthesis of cystine from a 3-carbon compound agrees well with the incorporation of sulfur by *Past. multocida*(16).

Summary. *Past. multocida* oxidizes pyruvate according to the Krebs cycle model. *Iso*-citrate dehydrogenase, aconitase, succinic dehydrogenase, fumarase and malic dehydrogenase were demonstrated with cell-free extracts or disrupted-cell preparations. The organism synthesizes aliphatic and aromatic amino acids from pyruvate carbon atoms. Formate carbon was incorporated into the serine-group of amino acids, alanine, the Krebs cycle intermediates and related amino acids, and nucleic acids.

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Development of Lactic Dehydrogenase Isozymes in the Chick Embryo.* (28960)

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Cells cultured *in vitro* undergo extensive ultrastructural(1) and histochemical(2,3) alterations. Among the histochemical alterations is the acquisition of a common electrophoretic pattern of lactic dehydrogenase (LDH) isozymes by organs which exhibited different patterns of isozymes prior to culture (3). In contrast, cells which have been grown on the chick chorioallantoic membrane (CAM) exhibit morphological characteristics not unlike those of the same cells cultivated *in vivo*(1). However, it has not been determined if the apparent morphological stability of CAM cultures is accompanied by stability of isozymes.

In the present study the LDH isozymes of

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developing liver, spleen and brain were demonstrated after starch gel electrophoresis. The isozymic patterns (zymograms) of LDH from these organs served as a basis for comparison with the LDH zymogram of liver and brain transplanted to the CAM for a period of 7 days. The results obtained provide information about the normal development of LDH isozymes as well as the stability of isozymes in CAM cultures.

Materials and methods. To establish the normal pattern of development of lactic dehydrogenase, the LDH fractions of 36 and 48 hour (incubation age) White Leghorn chick embryos and of brain, spleen, liver, CAM and whole blood from embryos of various ages were demonstrated. For the study of the effect of CAM transplantation on the tissue zymograms, small samples (about 5 mm square) of either brain or liver were dissected aseptically from 10 day chick embryos and transferred directly to the surface of the CAM of 10 day chick hosts through a window in the egg shell. This window was sealed with scotch tape and the egg incubated for an additional 7 days. At the end of the culture period, the transplant was freed from the adhering CAM and utilized for demonstration of the LDH isozymes. All tissues were homogenized in an equal volume of distilled water, subjected to repetitive freezing and thawing and centrifuged to remove tissue debris. The supernatant was then subjected to starch gel electrophoresis as previously described(4,5).

Following electrophoresis, LDH activity was demonstrated by immersing the starch columns for 1 hour at 37°C in a substrate containing 0.25 M Tris-HCl buffer; 0.1 M lactic acid; nicotine adenine dinucleotide (NAD), 0.3 mg/ml; phenazine methosulfate (PMS), 20 μg/mg; and Nitro BT, 0.5 mg/ml. The final pH of the solution was 7.2.

Results. Organs from chick embryos exhibited a total of 6 LDH isozymes with a maximum of 5 fractions present in any single organ (Fig. 1-2). For purposes of description the fractions were numbered 1-5 according to the system of Apella and Markert(6). In this system the most anodal migrating fraction is designated as LDH-1 while the isozyme which either exhibits the least mobility or which is most cathodal migrating is termed LDH-5. In the present study LDH-5 was further subdivided into LDH-5a and -5b in order to retain this nomenclature even though 6 isozymes were demonstrated (Fig. 2E).

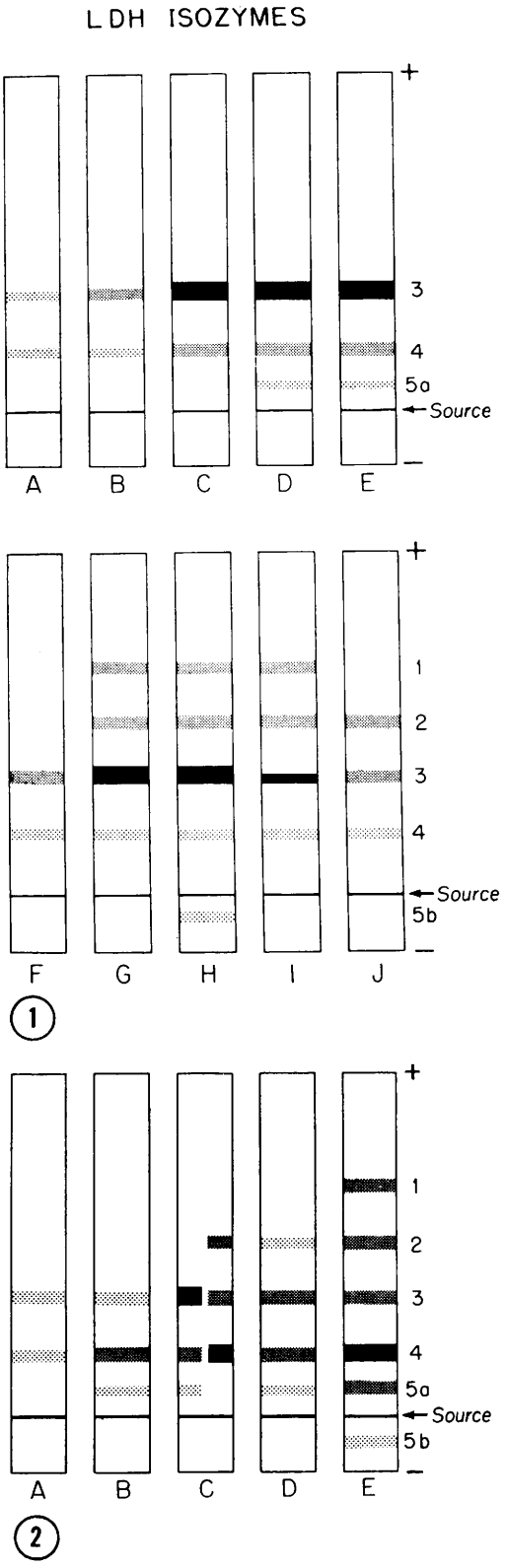
Two isozymes, LDH-3 and LDH-4, were the minimum number demonstrated. These isozymes were found in the tissues of young embryos (Fig. 1A, B) and in whole blood (Fig. 2A) at all ages studied. LDH-3 and LDH-4 were the only isozymes present in liver, spleen, and brain (Fig. 1C, F) until the 6th, 10th and 12th day of development, respectively.

An additional isozyme, LDH-5a, appeared in the spleen on the 12th day and in the brain on the 15th day (Fig. 1D). Three fractions, LDH-3, -4, and -5a, were found in the brain and spleen during the remainder of development and also after hatching (Fig. 1E).

Liver LDH isozymes exhibited a more complex developmental pattern. On the 7th day, 2 additional enzymes appeared, LDH-1 and LDH-2 (Fig. 1G) followed by a final enzyme, LDH-5b, on day 12 (Fig. 1H). This pattern was observed in the liver until after hatching when LDH-5b could no longer be demonstrated. Thus, the post-hatching zymogram of chick liver exhibited 4 isozymes, LDH-1, -2, -3 and -4 (Fig. 1I).

The zymogram of brain transplants was unchanged from that of the control tissue in that LDH-3, -4 and -5a were present. However, the zymogram of liver transplants differed from that of normal liver, either embryonic or after hatching. In this zymogram only 3 isozymes were found, *i.e.*, LDH-2, -3, and -4 (Fig. 1J). This pattern differed from the zymogram of embryonic liver of corresponding age by the absence of LDH-1 and -5b (Fig. 1H). The zymogram of the transplant differed from that of the post-hatched liver by the absence of one enzyme, LDH-1.

Since a pattern of 3 isozymes was found in both the transplants and the chorioallantoic membrane (CAM) (Fig. 2B), samples of CAM and liver transplants were demonstrated in parallel (Fig. 2C) and after mixing (Fig. 2D) to reveal either similarities or differences in the 3 fractions. These zymo-



grams demonstrated that the CAM isozymes were LDH-3, -4 and -5a, the same as those found in brain and spleen and in brain transplants. Thus the CAM and liver transplants differed by at least one enzyme, LDH-2 (liver) and LDH-5a (CAM) and were similar with respect to LDH-3 and LDH-4. To determine whether the 6 isozymes were electrophoretically distinct, homogenates of 17 day liver and CAM were pooled and subjected to electrophoresis. Examination of the zymograms of the pooled homogenate revealed 6 isozymes to be present (Fig. 2E) thus confirming that the electrophoretic mobility of each LDH fraction is a distinctive property of that fraction.

The presence of 6 isozymes suggested that one or more of the isozymes contained endogenous NAD or diaphorase(4,7) which accounted for the altered electrophoretic migration. This hypothesis was tested by omitting NAD and/or PMS from the enzyme substrate in order to observe whether reduction of the tetrazolium salt occurred. It was found that both NAD and PMS were essential for demonstration of all isozymes in the organs studied.

Discussion. Apella and Markert(6) followed by Cahn *et al*(8), were able to account for the 5 LDH isozymes observed in many tissues. However, neither group of investigators attempted to explain the number of isozymes in excess of 5 which have been reported(3,5,9,10). While it was previously suggested that the demonstration of more than 5 LDH isozymes was artifactual(8), Fritz and Jacobson(7) proposed that endogenous components of the respiratory chain were responsible for the altered electrophoretic mobility of the 5 principal LDH isozymes.

FIG. 1. Starch gel zymograms of LDH isozymes. (A, B) 36- and 48-hour embryos, respectively, (C) brain, days 3-12; spleen, days 8-10, (D) brain, days 15-21; spleen, days 12-21, (E) brain and spleen, days 1-10 post-hatching (F) liver, days 5-6 (G) liver, days 7-10 (H) liver, days 12-21 (I) liver, 1-10 days post-hatching (J) liver transplant.

FIG. 2. Starch gel zymograms of LDH isozymes. (A) Whole blood, pre- and post-hatching (B) chorioallantoic membrane (CAM), day 17 (C) CAM (left), liver transplant (right) (D) pooled homogenate of CAM and liver transplant (E) pooled homogenate of CAM and liver, day 17.

On the other hand, Boyer *et al*(11) have demonstrated that an allelic variant of one of the LDH subunits (LDH-1) may also result in more than 5 LDH isozymes. In the present study 6 LDH fractions were demonstrated (Fig. 2E). All of these fractions required both NAD and PMS for their demonstration indicating that neither active endogenous NAD nor diaphorase was present. Results obtained in this laboratory and reported also by Boyer *et al*(11), refute the claim of Cahn *et al*(8), that dilution of the sample is responsible for differences in the LDH pattern except as excessive dilution provides for an undemonstrable quantity of the less prevalent isozymes.

Two isozymes, LDH-3 and -4, were present at the earliest stages studied. LDH-1 and -2 appeared in the liver (Fig. 1G) on the 7th day of development followed by LDH-5a in the spleen (Fig. 1D) and LDH-5b in the liver on the 12th day (Fig. 1H). If the acquisition of additional isozymes indicates the onset of maturation, as it well may, then maturation was initiated in the order: (1) liver (entoderm), (2) spleen (mesoderm) and (3) brain (ectoderm). The additional change which occurred in liver at hatching (Fig. 1I) probably is a reflection of the change in functions which are peculiar to this organ and not to brain or spleen.

The fact that LDH-3 and -4 were the first isozymes present suggests near random assortment(12,13) of the LDH subunits. In later stages there was a slight increase in the predominance of the LDH-5 type of subunits in brain and spleen. In developing liver, the shift is from random assortment to a marked increase in the LDH-1 subunits resulting in the post-hatch liver zymogram of LDH-1, -2, -3, and -4 (Fig. 1I). Lindsay(12) has postulated that the LDH-1 and LDH-5 subunits are characteristic of tissues with high and low respiratory rates, respectively. If this is true, then the changes observed in this study indicate an unspecialized metabolic pattern during early development. However, with the onset of maturation the synthesis of isozymes is altered in a pattern which is characteristic of the organ undergoing maturation. The metabolism of liver is shifted toward a higher respiratory rate while brain and spleen remain unchanged.

While Lindsay has hypothesized the synthesis of LDH-1 and LDH-5 subunits by 2 different cell populations, with transfer of subunits from one population to the other, Boyer *et al*(11) have shown that 2 genetic loci in a single population can account for the heterogeneity of LDH. The random synthesis of isozymes followed by a characteristic alteration of the pattern, observed in the present study, supports the concept of multiple loci in a single cell. In fact, while 2 loci may be responsible for the synthesis of 2 types of subunits, other loci may be concerned with the assembling of the subunits. This view is supported by the fact that alteration of synthesis alone cannot account for the changes observed.

Although Lindsay(12) noted shifts in chick embryonic isozymes from cathodal fractions to anodal fractions, Philip and Vesell (3) reported the opposite shift. Both groups of investigators employed modifications of the original starch-gel method(14) which may account for the discrepancy in their results.

Philip and Vesell(3) reported a change in isozyme content of cells grown in tissue culture. This change was in the form of the development of an isozyme pattern common to all tissues. In chick liver, this change was in the form of a decrease in the anodal migrating isozymes. In the present study, a similar change was noted in liver transplanted to the chorioallantoic membrane. After 7 days in culture, only isozymes LDH-2, -3 and -4 were present (Fig. 1J). This zymogram differed from *in vivo* developing liver by the absence of LDH-1 and -5b and suggests a return to a randomly assorted zymogram as found in immature tissues. Brain transplants, in contrast, did not differ from control tissues; perhaps, because the isozyme pattern was already one of random assortment. Whether or not the change observed in liver transplants is a dedifferentiation as suggested (3) depends on the nature of the change and must be distinguished from a modulation.

Summary. It was observed that 6 LDH isozymes were present in chick embryonic

organs. Five of these isozymes occurred in developing liver while the 6th type was one of 3 isozymes found in brain and spleen. Brain and spleen exhibited only slight changes in isozyme content during maturation while more extensive changes occurred in liver. The demonstration of LDH isozymes of liver transplanted to the chorioallantoic membrane revealed that there was a reduction in the number of isozymes suggestive of a return to a less differentiated state.

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Factors Affecting Hemagglutination by Enteroviruses. (28961)

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The initial observation that the prototype strains of ECHO virus types 3, 6, 7, 11 and 12 and of Coxsackie virus group B type 3 could agglutinate human erythrocytes(1) was followed by other reports(2,3,4,5,6,7) in which the property of hemagglutination was described for prototype or non-prototype strains of ECHO virus types 13, 19, 20, 21 and 25, Coxsackie virus group A types 7, 20, 21 and 24, and Coxsackie virus group B types 1 and 5. Since JV-10 virus, known variously as Enterovirus type 59(8), Unclassified Picornavirus type 1 (U.S.)(9) or ECHO virus type 29(10) also has been found to hemagglutinate(11), at least one strain of 18 of the 59 enterovirus serotypes recognized in 1962(8) had been found to possess this biological property. Although hemagglutination has been reported for still other enterovirus serotypes(12,13,14), those mentioned above are the only ones for which it was demonstrated clearly that the hemagglutination reaction was specifically inhibited by homologous antiserum. The hemagglutination-inhibition (HI) test has been used successfully for virus identification and for demonstrating serologic responses following infection with hemagglutinating serotypes(6,7,13, 15,16,17,18,19,20,21).

To define further some of the factors that influence hemagglutination by enteroviruses, we studied representative strains of nearly all of the human enterovirus serotypes known to hemagglutinate as well as hemagglutinating strains of newly recognized, but as yet unclassified, enteroviruses. The results of these investigations are the subject of this report.

Materials and methods. Virus strains. Hemagglutinating enteroviruses studied included

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