SUMMARY

1. The behaviour of serum albumin and γ -globulin labelled with ^{131}I has been investigated in the isolated perfused rat liver.

2. Such a liver has been found capable of preferentially removing and then breaking down very small proportions of altered protein molecules present in preparations of plasma proteins isolated by the usual methods. The proportions of such altered molecules actually present have been found to vary widely according, at least in part, to the methods used for the preparation of each protein.

3. Very rapid breakdown occurred when human albumin was presented to the liver as part of an antigen-antibody complex.

4. On the other hand, very slow liberation of ^{131}I was found if the [^{131}I]proteins were first injected into rats and then, 48 hr. later, the plasma of these animals was used as the dose material for the liver perfusions. No difference could be detected between the behaviour of rat and human albumins which had been thus pretreated.

5. The observed rate of catabolism of rat albumin if pretreated as above was found to be very low. This suggests that in the rat the liver is responsible for not more than about 10 % of the total breakdown of serum albumin which occurs *in vivo*.

6. The proportions of these proteins which were retained in the liver after 5 hr. perfusion and after finally perfusing out with saline have been measured.

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Glutamic Dehydrogenase in the Developing Chick Embryo

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The processes of morphogenesis and differentiation in embryos may be expressed on the molecular level as an increasing complexity of arrangement of proteins in the embryonic cell. This arrangement may be accompanied by development or loss of certain enzymic activities during differentiation until the complement of enzymes characteristic of adult tissue is attained. Boell (1955) has reviewed the considerable amount of evidence that enzyme development is linked with functional differentiation during development.

The ontogenic sequence of carbohydrate and protein as primary energy sources during develop-

ment of the chick embryo (Needham, 1931) may be accompanied by the appearance of enzymes concerned with the conversion of carbohydrate into protein precursors. Glutamic dehydrogenase (GDH) plays an interesting role in amino acid metabolism, by providing a possible pathway for the production of glutamic acid from carbohydrate sources by the fixation of ammonia, or as an 'L-amino acid oxidase' in conjunction with the transaminases (Braunstein & Bychkov, 1939; Braunstein & Azarkh, 1945; Meister, 1955).

GDH activity has been measured in blastoderms, embryos and their extra-embryonic tissues during

development of the chick embryo, and the levels of enzyme activity compared with those of the 9-week-old hen. A preliminary report of this work has been presented (Solomon, 1956).

EXPERIMENTAL

Materials. α -Oxoglutaric acid (British Drug Houses) was recrystallized twice from hot acetic acid. Reduced diphosphopyridine nucleotide (DPNH) and a solution of purified ox-liver GDH were supplied by C. F. Boehringer and Soehne, Mannheim.

Tissues. Eggs were obtained from Rhode Island Red $\times$ Light Sussex fowls (Agricultural Research Council, Compton, Berkshire), and were incubated at 37° in a Westermette incubator. After varying periods of incubation, embryos and their extra-embryonic tissues were separated from the egg yolk, placed in ice-cold isotonic saline and their morphological development was classified according to the stages proposed by Hamburger & Hamilton (1951). The average incubation times quoted by these authors for each stage were used so that morphological development is expressed as 'corrected incubation time'. Blastoderms were removed whole until 35 hr. (10 somites stage) when the opaque area was dissected from the embryo. When the opaque area became vascular, it was subdivided into the vascular area (limited by the *sinus terminale*) and the outer periphery of the yolk sac, until the vascular area completely surrounded the yolk (at about 7 days of development). In later stages the total extra-embryonic tissue was separated into the yolk sac and allantoic membrane. The tissues studied were the blastoderm (embryo with opaque area), the embryo from 35 hr., the vascular area (35 hr.–6 days), total yolk sac (from 35 hr.) and the allantoic membrane from 6 days of incubation. Tissues were stored at -15°.

Assay of enzyme activity. The method used was essentially that of Olson & Anfinsen (1952), with the addition of nicotinamide and cyanide to the reaction mixture (Hogeboom & Schneider, 1953). The reductive amination of α -oxoglutaric acid in the presence of NH_3 and homogenate is accompanied by the oxidation of DPNH, which can be measured by the decrease in optical density at 340 m μ . The reaction mixture contained 1.6 μ moles of KCN, 1.2 m-moles of nicotinamide, 1.5 μ moles of DPNH (sodium salt), 0.1 M phosphate buffer, pH 7.6 (2.5 ml.), 60 m-moles of NH_4Cl and 0.1–0.2 ml. of enzyme solution or homogenate in each cuvette; total volume was 3 ml. Measurements were made in a spectrophotometer (Unicam SP. 500) at room temperature (20°). The optical-density reading for the blank was set at 0.4, and at zero time 10 μ moles of sodium α -oxoglutarate was added to the other cuvette and mixed by stirring with a spatulate glass rod. Readings of the decrease in optical density at 340 m μ against the blank were made every 30 sec. for 3 min. During this period the rate of oxidation proceeded linearly and the amount of DPNH oxidized was directly proportional to the concentration of the homogenates. All assays were made in duplicate. Hogeboom & Schneider (1953) have shown with mouse-liver homogenates that the amount of DPNH oxidized under similar conditions corresponded stoichiometrically with the amount of glutamic acid formed. One unit of GDH activity is defined as that amount which causes 1 μ mole of DPNH to be oxidized in 1 min., $\epsilon = 6.22 \times 10^6$ (Horecker & Kornberg, 1948) being used as the molecular extinction coefficient of DPNH.

Protein determination. The method of Sutherland, Cori, Haynes & Olsen (1949) was used. Bovine plasma albumin (Armour Laboratories) dissolved in 0.1 M-phosphate buffer, pH 7.6, was used as standard protein. Estimations were made within the range 40–200 μ g. of albumin in 5.5 ml.

pH optimum. All assays were made in 0.1 M-phosphate buffer, pH 7.6, which was found to be the optimum pH of GDH in an homogenate of 9-day-old chick embryos.

Homogenates. Copenhaver, McShan & Meyer (1950) and Hogeboom & Schneider (1953) found that GDH activity was greater when tissues were homogenized in water instead of in isotonic sucrose solutions. All homogenates in this work were made in water with a ground-glass homogenizer, and assayed immediately. In the early stages of development tissues from about six eggs were pooled for each homogenate; in later stages, tissues from two eggs were used. GDH activity was stable in frozen homogenates (-15°) for 24 hr.

Attempts to release further activity from homogenates. Hogeboom & Schneider (1953) have shown that GDH of mouse liver is localized exclusively in the mitochondria, and these authors obtained a threefold increase of activity after disruption of the mitochondria. When homogenates of hen liver were rapidly frozen and slowly thawed no increase in GDH activity occurred. Acetone-dried powders, prepared from hen liver by a method described by Morton (1955), showed less activity/mg. of protein than the original homogenate.

Enzyme concentration. When different concentrations of purified ox-liver GDH (Boehringer) were assayed, a linear relationship was found between decrease in optical density and concentration of enzyme within the range 0.04–1.6 $\times 10^{-3}$ units (1–16 μ g. of protein). Homogenates were assayed within this range. Olson & Anfinsen (1952) obtained a maximal specific activity of 30 units/mg. of protein, which is 300 times greater than that of the Boehringer preparation.

Reversibility of the reaction. Olson & Anfinsen (1952) reported that, when glutamic acid was used as a substrate in the presence of oxidized diphosphopyridine nucleotide (DPN^+), the values for specific activities of GDH preparations were one-tenth of those for the reverse reaction. This has been confirmed on homogenates of hen liver when DPN^+ -reduction values were one-fifth of those for DPNH oxidation. In view of the very low activities of GDH found in the tissues studied only the DPNH-oxidation reaction has been used for assay purposes.

Inhibitors. GDH is highly specific for L-glutamic acid (Euler, Adler, Günther & Das, 1938; Dewan, 1938; Olson & Anfinsen, 1953). The addition of L-glutamic acid (end product of the DPNH-oxidation reaction) to homogenates at twice the molar concentration of the substrate produced 50% inhibition of activity in 50 μ g. of purified GDH (Boehringer) and 80% inhibition of activity when it was present at ten times the molar concentration of the substrate. The addition of L-glutamic acid (ten times molar concentration of substrate) to a homogenate of hen liver (0.9 mg. of protein) produced 60% inhibition of GDH activity. Vallee, Adelstein & Olson (1955) have shown that GDH contains 4–5 g. atoms of Zn/mole of enzyme (mol.wt., 10^6). They found that activity was inhibited significantly by the metal-chelating agents sodium diethyldithiocarbamate and 1:10-phenanthroline. GDH activity of the Boehringer preparation (100 μ g.) was inhibited 50–80% by sodium

diethyldithiocarbamate (1 μ mole) and $\alpha\alpha'$ -dipyridyl (1 μ mole). Significant inhibition of GDH activity was produced when either of these two compounds (0.1 μ mole) was added to a homogenate of livers from 14-day-old chick embryos (corresponding to 0.48 mg. of protein).

RESULTS

Egg yolk is known to contain many enzymes and it was not surprising to find GDH present in unincubated egg yolk of the fowl. Yolks obtained from eggs containing developing embryos showed a considerable increase of activity from the second day to 7.5 days of incubation (Table 1), and a decrease during the period 7.5–18 days of incubation. This behaviour is similar to that observed by Remotti (1927) for a protease having maximum activity in fowl-egg yolk at the tenth day of incubation, which is the time of maximum protein catabolism in the chick embryo (Needham, 1931). No GDH activity has been detected in egg white.

The total GDH activity of the chick embryo, the yolk sac and the vascular area of the yolk sac is

Table 1. *Changes of GDH activity in egg yolk during development of the chick embryo*

Incubation period (days)	$10^3 \times$ GDH activity	
	(Units/yolk)	(Units/mg. of protein)
0	1130	0.32
1	1240	0.35
2	1140	0.63
7.5	4000	2.0
11	3600	1.0
18	600	0.47

Fig. 1. Levels of GDH activity in the chick embryo and the yolk sac during development. ●, Embryo; △, vascular area; □, yolk sac.

shown in Fig. 1, a semi-logarithmic plot being used for GDH activity. Activity was first detected in blastoderms, where it increased more rapidly than in the embryo until about the third day of incubation. This activity was not localized in the opaque area, as the vascular area showed the same rate of increase up to 3 days of incubation. After the third day of incubation GDH activity increased less rapidly until the yolk sac was entirely vascular (about the seventh day). The rate of increase of GDH activity in the total yolk sac in later stages was very much less rapid. GDH activity was first detected in the chick embryo at the 10 somites stage (35 hr. of incubation) and activity increased rapidly until the 4.5 days developmental stage, when a change in the rate of increase was observed. A similar change in growth rate (on a wet wt. basis) has been observed at this stage of development.

The distribution of GDH in the egg during development is shown in Fig. 2. GDH in the yolk is not severely depleted until the 16th day, when the embryo contains nearly half the total GDH in the egg. The amount present in the extra-embryonic tissues (the allantoic membrane is not shown as its contribution to the total is negligible), although greater than that of the embryo until the tenth day, is never more than 5% of the total activity of the egg.

The GDH activities/g. wet wt. in the yolk sac and the vascular area of the yolk sac during 1–16 days of incubation are shown in Fig. 3. The activities/mg. of protein in the yolk sac follow a similar pattern to those relative to wet wt. except that the slight increase in activity/g. wet wt. at 4 days (Fig. 3) is accentuated on a protein basis (2.5 days, 0.6×10^{-3} ;

Fig. 2. Distribution of GDH in the hen's egg during incubation. ●, Embryo; ■, yolk sac; □, yolk.

4 days, 1.4×10^{-3} units/mg. of protein) and no sharp rise in activity (on a protein basis) was found in the vascular area of the yolk sac just before the third day of incubation. However, at 6.5 days of incubation two high results were obtained for the vascular area (average 1.7×10^{-3} units/mg. of protein).

In the allantoic membrane, GDH activity/g. wet wt. showed only a very slight increase from 6 to 18 days of incubation (6 days, 16.5×10^{-3} ; 11 days, 23×10^{-3} ; 18 days, 24×10^{-3} units/g. wet weight). The increasing protein content of the allantoic membrane during this period of incubation showed as a decrease of activity/mg. of protein (6 days, 1.27×10^{-3} ; 11 days, 0.51×10^{-3} ; 18 days, 0.48×10^{-3} units/mg. of protein).

The opaque area of blastoderms possesses a higher activity than any other extra-embryonic tissue at any time (on a wet wt. and protein basis) during the whole period of development; there is a small peak on the fourth day of incubation which may be due to the sudden rise in GDH activity (on a wet wt. basis only) in the vascular area just before the third day.

The relative GDH activities (per g. wet wt. and per mg. of protein) in whole chick embryos during development (Fig. 4) again follow a similar pattern. GDH activity on a wet wt. basis increases slightly from the 10 somites stage to the second day of incubation, followed by a fall until the fifth day. Activity remains constant until the tenth day, after which there is a steady rise up to 16 days of incubation, when the embryo has reached the high level of the 2 days of incubation stage. On a protein basis, GDH activity exhibits a more marked peak on the second day of development, but otherwise activity remains nearly constant during later development. This peak of activity/mg. of protein is of the same order as that found in the opaque area of blastoderms 24 hr. earlier (2.6×10^{-3} units/mg. of protein).

Fig. 3. Relative GDH activities in the yolk sac during development of the embryo. Δ , Vascular area of yolk sac; $\blacksquare$, yolk sac.

From the fourth day the constant level of activity/mg. of protein in the embryo is five times greater than that of the yolk sac.

The increase of GDH activity (per g. wet wt.) in whole embryos after 10 days was further studied by measuring GDH in organs and tissues of the embryo. Tissues studied were liver (from 7 days), brain (from 10 days), heart and skeletal muscle (from 14 days until 3 days after hatching). The GDH activities/g. wet wt. of these tissues during late development of the embryo and early development of the chick (Fig. 5) remain constant in the brain and heart, but there is a slow increase in the liver up to the 16th day of incubation and then a decrease until 3 days after hatching; a similar pattern occurs in skeletal muscle, with a peak at 18 days of incubation. On a protein basis a slightly different accumulation of activity is evident (Fig. 6); the activities of heart and brain increase steadily after 19 days of incubation, liver shows a rather earlier peak (14 days) and

Fig. 4. Relative GDH activities in the developing embryo. $\bullet$, GDH activity/mg. of protein; $\circ$, GDH activity/100 g. wet wt.

Fig. 5. GDH activity/g. wet wt. in tissues of the developing chick embryo. $\bullet$, Liver; $\circ$, muscle; Δ , brain; $\square$, heart.

Table 2. Comparison of GDH activities in organs from a 3-day-old chick and a 9-week-old hen

	$10^8 \times \text{GDH activity}$					
	(Units/g. wet wt.)		Increase (%)	(Units/mg. of protein)		Increase (%)
	Chick (3 days)	Hen (9 weeks)		Chick (3 days)	Hen (9 weeks)	
Heart	40	217	440	1.4	3.1	120
Liver	48	257	440	1.7	1.8	150
Brain	25	227	810	0.7	2.4	220
Muscle	173	515	200	1.8	10.3	470

Fig. 6. GDH activity/mg. of protein in tissues of the developing chick embryo. ●, Liver; ○, muscle; △, brain; □, heart.

Table 3. GDH activity per cell in early chick embryos

Corrected incubation time (hr.)	$10^{-6} \times \text{no. of cells}$	$10^{12} \times \text{GDH activity (units/cell)}$
34	1.0	240
36	1.2	220
47	4.3	93
53	8.2	61
56	12	58
60	15	53
70	92	15

skeletal muscle exhibits a nearly tenfold rise in activity from the 16th to the 18th day.

Leslie & Davidson (1951) have shown that there are large variations in cell mass and protein in the tissues of the chick embryo during development. The average cell number of a known weight of tissue was calculated from these authors' figures for deoxyribonucleic acid phosphorus ($2.35 \times 10^{-7} \mu\text{g./nucleus}$). The average GDH activity/cell in the developing tissues of the chick embryo presents a similar pattern to Fig. 6 except for muscle where GDH activity/cell continues to increase after the

18th day of incubation to 3 days after hatching. The decrease in muscle-GDH activity on a wet wt. and protein basis after the 18th day of incubation (Figs. 5 and 6) now appears to be due to the great increase in muscle-cell mass and protein known to occur during this period of development (Leslie & Davidson, 1951).

For comparison with levels of GDH activity of embryonic organs, GDH activity has been measured in the corresponding tissues of a 9-week-old hen. The general increase in activity with age is shown in Table 2. The percentage increases in GDH activity on a wet wt. basis are in the ascending order muscle, heart and liver, brain; whereas the order on a protein basis is heart, liver, brain and muscle. The heart and liver show the same percentage increases in activity, the brain increases its activity/unit wet wt. by eightfold and activity/mg. of protein is increased nearly fivefold in muscle.

It is possible to calculate average GDH activity/cell in embryos during the 35–72 hr. incubation period, on the basis of the average number of nuclei/embryo, which has been found to show a steady exponential increase during this period (Solomon, 1957). The results (Table 3) show a gradually diminishing amount of enzyme/cell in the embryo despite the sharp peak of activity/mg. of protein at 2 days (Fig. 4). This may mean that many cells in the later stages of development contain little or no enzyme, i.e. there may be a localization of enzyme activity.

The highest specific activity obtained for a preparation of ox-liver GDH is 1 unit/30 $\mu\text{g.}$ of GDH preparation (Olson & Anfinsen, 1952). This figure may be used to calculate the maximum amount of GDH present in any tissue; the highest level of GDH activity/mg. of protein is 10.3×10^{-3} unit in skeletal muscle of the 9-week-old hen, which corresponds to 0.03 % of the total protein. The maximum amount of GDH present in the 2-day-old chick embryo on this basis would be 0.01 % of the total protein.

DISCUSSION

The increase of GDH activity in egg yolk from the second to the eighth day of incubation may be due to the disruption of yolk particles, e.g. vitelline

platelets (Panijel, 1950), during the metabolism of the yolk. The comparatively large amount of GDH in the egg yolk during development is in excess of the requirements of the embryo until the pre-hatching stages; however, it is not yet known whether the GDH of the yolk is transferred to the embryo during later developmental stages or whether the embryo can synthesize GDH independently of the yolk.

The yolk sac is known to behave as a 'transitory liver' in respect to glycogen storage (Bernard, 1872) and as mature liver in its response to insulin (Zwilling, 1951). It is interesting to note that the high level of GDH activity/mg. of protein of blastoderms (2.6×10^{-3} unit) is greater than the activity of the yolk sac at 4 days (1.4×10^{-3} unit), vascular area at 6.5 days (1.7×10^{-3} unit) and the liver at 14 days (1.9×10^{-3} unit). The level of activity of 14-day-old chick embryo liver is identical with that of liver of the 9-week-old hen, and the liver at 14 days can be regarded as temporarily 'functionally mature' as far as GDH content is concerned. The high levels of GDH in the yolk sac during early development lend support to its suggested function as a 'transitory liver'.

The presence of minute amounts of GDH in tissues of the chick embryo does not indicate the extent to which the enzyme is being used for glutamic acid oxidation or synthesis. Both α -oxoglutaric acid (Westfall, Peppers & Earle, 1955) and glutamic acid (Rupe & Farmer, 1955) are known to be present in 9-day-old chick embryos and their extra-embryonic tissue. Rupe & Farmer (1955) consider that the large reserves of free glutamic acid in the egg yolk and white are sufficient to satisfy the requirements of the developing embryo and, as these authors point out, the amount of glutamic acid that might be synthesized by the embryo is necessarily small. The minute amounts of GDH found are in agreement with this suggestion, and may only act as a localized 'reserve mechanism' for ammonia fixation; however, Moog (1952) considers that chick embryonic tissues may not be provided with the enzyme reserves possessed by adult tissues.

When the daily increment of GDH accumulation relative to wet weight in the developing embryo is plotted, the curve shows some similarity with those of total carbohydrate and protein (Needham, 1931) in falling rapidly until the sixth day of development. After this time the GDH curve remains at a nearly constant level and resembles that of carbohydrate rather than protein, which rises rapidly after the seventh day of development.

Certain other enzymes which have been studied during the development of the chick embryo show some similarities to GDH. Levy & Palmer (1940) found that dipeptidase in the chick embryo had a high level of activity (on a wet weight basis) at

1.5 days of incubation, then activity decreased until the third day, and remained at a constant low level until the ninth day before rising to its former high level on the 16th day. This is a similar pattern to that of GDH after 2 days of incubation. The peak of GDH at 2 days precedes that of aminopeptidase (Levy & Palmer, 1943) at 2.5 days of development, which then follows a pattern similar to that of GDH during the later developmental stages. It appears that these peaks of activity in the early chick embryo follow the ontogenic sequence, dipeptidase, GDH and aminopeptidase, during 1.5-2.5 days of incubation. These peaks of activity in early embryos were not found for glutamotransferase and glutamine synthetase (Rudnick, Mela & Waelsch, 1953), which increase *pari passu* with total protein in the embryo and yolk sac (as did GDH after the fourth and sixth day respectively). Transferase activity (per mg. of protein) increased steadily in the liver between the sixth and 18th day of incubation but there was no peak of activity as with GDH. The change in GDH activity (per mg. of protein) in developing skeletal muscle shows a marked resemblance to that of adenosine triphosphatase (Moog, 1947), which increased threefold between the 12th and 20th days and then decreased to a constant level at 3 days after hatching. Moog (1952) states that the peak of adenosine triphosphatase activity coincides with muscular activity before hatching; the sharper increase (tenfold) in GDH activity in muscle during the 14th-18th day may also be associated with muscular activity of the embryo.

The levels of GDH (per mg. of protein) in tissues during development of the chick embryo are generally lower than in tissues of the 9-week-old hen. However, with GDH it has been shown that the opaque area of the blastoderm and the 2-day-old embryo have GDH activities as high as those of adult brain, heart and liver. The ratio of minimum to maximum activities in ascending order is chick embryo and allantoic membrane (3), heart (6), liver (7), brain (10), yolk sac (13) and muscle (26).

The GDH activity of early blastoderms is presumably localized in the opaque area since embryos of 10 somites have only traces of enzymic activity. The high level of activity (per mg. of protein) in the opaque area of the blastoderm rapidly falls with development and is followed by a surge of activity in the whole embryo at 2 days. It appears that the opaque area contains a high level of GDH during the formation of the primitive streak and early somites; a measurable amount of the enzyme occurs in the embryo after 24 hr., where it may have an important localized function in the morphogenic processes at this stage. The next surge of activity per mg. of protein occurs in the yolk sac at 4 days, but is not followed by a surge in total embryonic activity and may be connected with the mobilization of GDH in

the yolk. On the 14th day there is a pronounced surge in the liver which precedes a greater surge of activity in skeletal muscle on the 18th day. These surges of activity probably represent a pattern of functional differentiation of certain cells in the embryo, and in its liver and muscle.

SUMMARY

1. An increase of glutamic dehydrogenase (GDH) activity in yolk of the hen's egg has been shown to occur in the mid-incubation period.

2. Generally, the levels of GDH in embryonic tissues are less than those of tissues of the hen except for the blastoderm and 2-day-old embryo, which have GDH levels as high as those of some hen tissues.

3. GDH has been found to increase *pari passu* with total protein in the whole embryo and yolk sac from the fourth and sixth day of incubation respectively.

4. GDH activity in the chick embryo and its extra-embryonic tissues during development has been shown to have similarities with certain other enzyme activities.

5. In certain tissues of chick embryos during later developmental stages considerable surges of activity are found to occur which may be associated with functional differentiation.

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Interrelationships between Lysine and $\alpha\epsilon$ -Diaminopimelic Acid and their Derivatives and Analogues in Mutants of *Escherichia coli*

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The little that is known of the biosynthesis or metabolism of lysine in bacteria suggests that the biosynthetic pathway is different from that in other species (for review see Work, 1955; Strassman, Thomas & Weinhouse, 1956). *meso*- $\alpha\epsilon$ -Diamino-

pimelic acid, which is converted into L-lysine by enzymic decarboxylation (Dewey, Hoare & Work, 1954), has now been established as a lysine precursor in some bacteria. We have investigated further relationships between lysine and diaminopimelic acid by studying the growth of certain diaminopimelic acid- and lysine-requiring mutants derived from *Escherichia coli* 9637 (Davis, 1952)

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