UNIVERSITY MICROFILMS, ANN ARBOR, MICH.

L. C. Card no. Mic 58-1450

POPP, Raymond Arthur. COMPARATIVE METABOLISM OF BLASTOCYSTS, EXTRAEMBRYONIC MEMBRANES, AND UTERINE ENDOMETRIUM OF THE MOUSE WITH REFERENCE TO INTERDEPENDENT METABOLIC ACTIVITIES OCCURRING DURING EMBRYOGENY.

University of Michigan, Ph. D., 1957
Zoology

Negative microfilm received from university.

COMPARATIVE METABOLISM OF BLASTOCYSTS, EXTRAEMBRYONIC MEMBRANES, AND UTERINE ENDOMETRIUM OF THE MOUSE WITH REFERENCE TO INTERDEPENDENT METABOLIC ACTIVITIES OCCURRING DURING EMBRYOGENY

by

Raymond Arthur Popp

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in the
University of Michigan
1957

Doctoral Commitee:

Associate Professor Clement L. Markert, Chairman
Assistant Professor John M. Allen
Assistant Professor Karl F. Guthe
Assistant Professor James F. Hogg
Assistant Professor Norman E. Kemp

COMPARATIVE METABOLISM OF BLASTOCYSTS, EXTRAEMBRYONIC MEMBRANES, AND UTERINE ENDOMETRIUM OF THE MOUSE WITH REFERENCE TO INTERDEPENDENT METABOLIC ACTIVITIES OCCURRING DURING EMBRYOGENY

By Raymond Arthur Popp

The objectives of this investigation were to study various aspects of the metabolism of early mouse embryos, extraembryonic membranes, and uterine endometrium and to postulate from these studies possible interdependent and interrelated metabolic activities occurring among these tissues that may have significance in embryonic development. The nature of the cellular outgrowth arising from tissue explants of mouse blastocysts, extraembryonic membranes, and uterine endometrium was studied in tissue culture. Various media and culture vessels were used in an attempt to promote growth of the fastidious blastocysts. The results demonstrated that aerobic environments are more hostile than anaerobic environments to young embryos; that the morphogenetic substances, present in vivo but apparently absent in vitro, are very labile; and that a dynamic steady state, which governs the oxidation-reduction potential, may be an important growth-promoting factor in vivo, which has not been duplicated as yet in vitro.

Uniformly labeled glucose (U-glucose-C-1^4) and U-fructose-C-1^4 were added to the culture media of four

day old blastocysts, extraembryonic membranes (decidua, amnion, chorion frondosum, allantois, visceral yolk sac, and placenta) explanted at various stages of development, and uterine endometrium explanted at estrus and also at four and one-half days after copulation. The metabolites formed from U-glucose-C-14 and U-fructose-C-14 were separated into two fractions---an alcoholic extract and an alcoholic precipitate. After appropriate pre-treatment the metabolites in each fraction were separated and identified by two dimensional paper chromatography. The radioactive metabolites were assayed both by autoradiography and with the aid of a geiger counter. The identified metabolites containing radioactive carbon derived from radioisotopically labeled glucose and fructose were lactate, pyruvate, succinate, alanine, serine, glycine, glutamate, aspartate, proline, histidine, cysteine, and an unknown ninhydrin-positive substance. These results were discussed in the light of probable biosynthetic pathways. The data indicated that mouse blastocysts in vitro metabolized anaerobically, whereas all the other tissues demonstrated both aerobic and anaerobic metabolism in vitro. The patterns of metabolism in vitro were used as a basis for postulating possible interdependent and interrelated metabolic activities occurring among these tissues in vivo. The yolk sac, which is vestigial in most mammals, was metabolically very active in the mouse even during the later periods of gestation and probably contributes significant amounts of nutrients for the fetus

throughout gestation. It was formerly believed that the yolk sac became physiologically vestigial following the development of the chorioallantoic circulation.

The relative quantities of amino acids derived from U-glucose-C-14 and U-fructose-C-14 metabolism were very different. The data demonstrated that the quantities of serine and, usually, glycine synthesized from U-fructose-C-14 were relatively greater than those from U-glucose-C-14, suggesting the existence of an additional previously unrecognized pathway for serine synthesis from a carbohydrate source.

ACKNOWLEDGEMENTS

The writer is grateful to Professor Clement L. Markert for his inspiration and encouragement during the course of the research and for his helpful assistance in the preparation of this dissertation. Sincere appreciation is also due to all the members of his committee, especially Professor James F. Hogg for his careful evaluation of the biochemical aspects of the dissertation. He also wishes to express his appreciation to the Horace H. Rackham School of Graduate Studies for the financial assistance provided by the Graduate Student Research Fund for purchasing the radioactively labeled isotopes used in this investigation and also for the Horace H. Rackham Predoctoral Fellowship received for his last year of study. He is also grateful to all the members of the Zoology Department for their consideration and helpfulness and to his wife and friends for making his graduate training both enjoyable and rewarding.

TABLE OF CONTENTS

		Page
I.	INTRODUCTION	1
II.	HISTORICAL REVIEW	8
III.	MATERIALS AND METHODS	18
IV.	RESULTS	32
	In Vitro Culture of Blastocysts	32
	Growth and Metabolism of Mouse Blastocysts In Vitro	36
	Growth and Metabolism of Uterine Endometrium In Vitro	38
	Growth and Metabolism of Decidua In Vitro	39
	Growth and Metabolism of Placenta In Vitro	40
	Growth and Metabolism of Allantois In Vitro	41
	Growth and Metabolism of Chorion Frondosum In Vitro	42
	Growth and Metabolism of Amnion In Vitro	42
	Growth and Metabolism of Visceral Yolk Sac In Vitro	43
V.	DISCUSSION	50
	The Nature of the Factors Promoting Blastocyst Growth	50
	Blastocyst Metabolism	54
	Uterine Endometrium	55
	Decidua	57
	Placenta	59
	Allantois	60
	Chorion Frondosum	61
	Amnion	63
	Visceral Yolk Sac	64
	Biochemical Aspects of Embryonic Metabolism	67
VI.	SUMMARY	75
APPENDIX		78
LITERATURE CITED		92

LIST OF TABLES

Table		Page
1.	Synthetic Medium No. 703	20
2.	Cellular Outgrowth Obtained from Tissue Explants	46
3.	Labeling of Amino Acids from Radioactive Carbohydrates (Qualitative Results)	47
4.	Labeling of Amino Acids from Radioactive Carbohydrates (Quantitative Results)	48-49

LIST OF FIGURES

Figure		Page
1.	Autoradiogram Illustrating the Purity of U-fructose-C-14	79
2.	Lactate Synthesized by Four Day Blastocysts from Radioactively Labeled Glucose	79
3.	Labeled Amino Acids Derived from Radioactively Labeled Glucose by Uterine Endometrium	81
4.	Labeled Amino Acids Derived from Radioactively Labeled Fructose by Nineteen Day Visceral Yolk Sac	81
5.	Labeled Amino Acids Derived from Radioactively Labeled Glucose by Nine and One-Half Day Decidua Capsularis	83
6.	Labeled Amino Acids Derived from Radioactively Labeled Fructose by Nine and One-Half Day Decidua Basalis	83
7.	Labeled Amino Acids Derived from Radioactively Labeled Glucose by Thirteen and One-Half Day Placenta	85
8.	Labeled Amino Acids Derived from Radioactively Labeled Fructose by Fourteen and One-Half Day Placenta	85
9.	Lactate Synthesized by Fourteen and One-Half Day Amnion from Radioactively Labeled Fructose	87
10.	Lactate and Succinate Synthesized by Nine and One-Half Day Visceral Yolk Sac from Radioactively Labeled Glucose	87
11.	Autoradiogram Illustrating the Amino Acids Formed by Nine and One-Half Day Visceral Yolk Sac from Radioactively Labeled Glucose	89

Figure Page

12. Chromatogram Illustrating the Corresponding Positions of the Labeled Amino Acids, as Well as the Unlabeled Amino Acids, Identified with Ninhydrin 89

13. Labeled Amino Acids Derived from Radioactively Labeled Glucose by Thirteen and One-Half Day Visceral Yolk Sac 91

14. Labeled Amino Acids Derived from Radioactively Labeled Fructose by Fourteen and One-Half Day Visceral Yolk Sac 91

INTRODUCTION

Research in embryology has progressed from the descriptive morphology of developing embryos to the more recent investigations of the physical and biochemical nature of development. Wilhelm Roux (1850-1924) was principally responsible for initiating the modern efforts to analyze development in mechanistic terms. He focused attention on the importance of analyzing the underlying physical and chemical forces which operate in biological systems.

Early in the twentieth century many researchers, notably Driesch, Spemann, and Mangold, successfully investigated the influence of some physical factors upon embryonic development. However, efforts to analyze the biochemical forces affecting embryogenesis were less successful and little was learned about the biochemistry of morphogenesis before 1930. In the next decade, as summarized by Needham in his report on the "Biochemical Aspects of Organizer Phenomena", investigations by Waddington, Pasteels, Holtfreter, and Lehmann advanced the "knowledge centering round the biochemical nature of the 'morphogenetic hormones' which act in normal embryonic development, forming a hierarchy of inductors." Needham goes on to say that "at the heart of morphogenetic processes there is a metabolic factor, not only because the proteins of the cell structure on which in the

last resort morphological architecture must be based are built up by enzymic reactions, but also because the stimulating substances, the morphogenetic hormones, are themselves chemical molecules with a metabolic origin and a metabolic fate." (Needham, 1940).

The importance of mechanistic analyses was realized much earlier by investigators in other fields of biology. Claude Bernard (1813-1878), the French physiologist, adopted this approach in his investigations of the physiology of nutrition, from which he formulated general concepts which also had embryonic significance. Perhaps Bernard's greatest discovery was to find that the liver builds up very complex substances which it manufactures from simpler substances brought to the liver in the blood. He showed that these substances are stored in the liver and are released when the body needs them. This discovery clearly showed that organs could synthesize complex substances as well as catabolize them. He introduced the idea of a "physiological synthesis", in which the functional activities of different organs in the body are interrelated and interdependent for the physiological unity of the body.

Since Bernard's discovery, many more examples have been found of interrelated and interdependent cellular activities. The developing embryo within the uterus exemplifies such activities. The appropriate uterine conditions necessary for the developing embryo cannot be provided without the harmonious interactions of the embryo, its extraembryonic

membranes, and the uterine endometrium. The present investigation was done to analyze some aspects of the metabolism of these interacting tissues and to study the nature of the harmonious interactions occurring among these tissues.

A rather specific interaction is known to exist between the uterus and embryo. In 1951 Chang found that the successful intraspecific transfer of rabbit ova was dependent upon equivalence in the sexual phase of the donor and recipient animals. Briones and Beatty (1954) showed that the interspecific transfer of early embryos almost always results in death of the embryos. Since early embryos do not produce antibodies, there must be a rather specific uterine environment for the embryos of each species. Thus, embryos are dependent upon the unique characteristics of the milieu in utero of their own species.

Histological studies show that the uterine epithelium hypertrophies just prior to the entrance of blastocysts into the uterine lumen from the oviducts. This hypertrophy is accompanied by an increase in the vascular supply and an elevated metabolism, which suggests that the uterus may be synthesizing nutrients for the blastocysts. There is a pronounced decidual proliferation immediately following blastocyst implantation. The decidua initially has a high content of glycogen, which subsequently is greatly reduced throughout embryogenesis. On the other hand, glycogen begins to appear in the visceral yolk sac about the tenth day of gestation and it reaches its highest concentration there by

the fifteenth day. It is believed that the glycogen in the decidua is an energy reserve which is absorbed and metabolized by the visceral yolk sac cells and supplies nutrients for the developing embryo (Wislocki et al., 1946).

The yolk sac is a highly specialized structure in the mouse. It develops to its greatest extent early in pregnancy. The apical ends of the cells in the visceral wall of the yolk sac lie in the yolk sac cavity during early embryogenesis. Beneath these cells are the developing blood vessels of the omphalomesenteric circulation embedded in the splanchnic mesoderm. In early pregnancy the yolk sac is separated from the uterine lumen by the decidua capsularis, parietal wall of the yolk sac, and Reichert's membrane. As pregnancy progresses, however, these membranes gradually disintegrate. About the fifteenth day of gestation the cells of the visceral wall of the yolk sac bathe directly in the fluids of the uterine cavity (Wislocki et al., 1946).

The chorio-allantoic placenta begins to develop about the tenth day of pregnancy. As the chorio-allantoic placenta develops, it gradually replaces the yolk sac as the principal organ of exchange. If one compares the functions of the yolk sac and the chorio-allantoic placenta of several different species, the yolk sac appears to become less important with the establishment of the allantoic placenta. The yolk sac generally becomes vestigial in species which possess a highly developed allantoic placenta (Mossman, 1937). Therefore, some investigators believe that the yolk

sac is physiologically vestigial, even though morphologically conspicuous, in the later periods of gestation in rodents (Dempsey, 1953).

The chorio-allantoic placenta of the mouse does not supply the fetus with nutrients until comparatively late in pregnancy. The chorio-allantoic circulation becomes functional about the eleventh day after fertilization. Thus, for over one-half of the period of gestation, which is nineteen to twenty days in the strain of mice used in these experiments, the embryos are nourished from other sources. Some evidence suggests that the embryos obtain much of their nutrition through the enlarged visceral yolk sac which is metabolically very active (Wislocki et al., 1946).

A great amount of thought has been given in recent years to the problems of the metabolism of mammalian eggs, early embryos, and the specialized extraembryonic structures, but no one has successfully analyzed the metabolism of mammalian eggs or blastocysts. It is unknown which of the metabolites in the uterine fluid can be absorbed and metabolized by the blastocysts. The exergonic reactions and the synthetic capacities of blastocysts have not been investigated. It is generally thought that the role of the extraembryonic membranes is mainly mechanical, but specific biochemical substances for the support of growth and differentiation in the embryo may also be provided by these membranes. An analysis of these problems has not been attempted because mammalian embryos at early stages of development are very small and

the quantity of metabolites is minute, thus making it very difficult to study the metabolism of early embryos by gross biochemical analyses.

Recent technological advances for assaying the metabolism of small quantities of tissue have enabled investigators to study problems today which were not feasible a decade ago. In particular, the development of chromatography for the separation and identification of a large number of biological substances and the availability of radioisotopically labeled metabolites of high specific activity to trace the pathways of metabolism make it possible now to study some aspects of the metabolic activities of mammalian blastocysts and of small bits of extraembryonic tissues, a study which could not have been done a few years ago. It is possible, for example, to determine the capacities of these tissues to convert uniformly labeled glucose-C-14 into amino acids and other metabolites. Those metabolites synthesized by the blastocysts and extraembryonic membranes from the labeled sugar can be, in part, separated and identified by two-dimensional paper chromatography; compounds which contain radioactive carbon must have been derived at least in part from the labeled sugar.

It has been known for some time that the transformation of one metabolite into another metabolite is catalyzed by tissue enzymes and that the enzymatic composition of tissues is governed by the genetic makeup of cells and by their developmental history. Thus, when an active metabolic

conversion of one compound into another is known to be governed by a specific enzyme or group of enzymes, the conversion becomes evidence for the activity of the enzymes required for the conversion. It must be kept in mind, however, that this study was done on isolated tissues in vitro and it may not reflect in all instances the metabolism of the tissues placed in their normal environment in their proper relationships with adjacent tissues in vivo.

HISTORICAL REVIEW

Ever since Heape (1890) successfully transferred some four-celled angora rabbit embryos into a Belgian hare from which two angora rabbits were later born, embryologists have been trying intraspecific and interspecific transfers of early embryos of many species. In 1933 Nicholas succeeded in the intraspecific transfer of rat embryos. The first successful transfer of mouse ova was reported by Bittner and Little (1937) in a study of the transmission of breast and lung cancers in mice. These results demonstrated that mammalian embryos could be removed from the oviducts and uteri without injury and, if placed in a suitable environment, would continue their normal development.

Since it was impossible to observe the growth and differentiation of mammalian embryos in utero, embryologists sought a medium which would permit in vitro observations of the developmental processes normally occurring in utero. Harrison (1908) and Carrel (1912) had shown that some embryonic tissues could be cultured in plasma clots. Their success stimulated embryologists to try to culture mammalian embryos in vitro in similar media.

In 1913 Brachet was able to grow five to seven day old rabbit blastodermic vesicles for about forty hours in plasma clots. Maximow (1923, 1925) found that there was an

increase in the size of the egg cylinder and an irregular growth of various structures when rabbit egg cylinders of six and one-half to seven and one-half days development were cultured in plasma clots for two to three days. He found that when the embryonic shields were removed from the egg cylinder and cultured in vitro for three to four days, a much better differentiation of the embryonic area resulted. Beating hearts, blood vessels, medullary tubes, and brain vesicles could be identified in these cultures. Although these experiments with rabbit embryos did offer some rewarding results, investigators had little success in culturing other mammalian embryos in vitro.

In view of the generally poor results obtained from the in vitro growth of early mammalian embryos in plasma clots, investigators began examining the ability of early embryos to grow and differentiate in other media. Nicholas and Rudnick (1933) found that the chorio-allantoic membrane of the chick embryo promoted the growth and differentiation of rat embryos explanted at eight and one-half to ten days of development. In some cases these explanted embryos developed a functional fetal placenta and differentiated into typical twelve day embryos, but they soon degenerated because of their dependence upon a maternal placental circulation. Younger embryos transplanted to the chorio-allantoic membrane failed to grow or differentiate. With a slightly modified technique, Gluecksohn-Schoenheimer (1941, 1944) showed that seven to nine day old mouse egg cylinders developed for

about three days when they were transferred into the extra-embryonic coelom of seventy-two to eighty hour chick embryos. Here again the younger mouse embryos did not grow or differentiate.

While some investigators (Nicholas and Rudnick, 1938) proceeded to do in vitro experiments with the egg cylinder and head fold stages of development, the most promising stages studied in plasma clot cultures, other investigators tried to determine what factors were controlling the growth of the blastocyst in utero. The general procedure used to determine the nature of the inductors, present in the uterus but absent in culture media, was to find substances which stimulated growth in utero and then to ascertain whether they also stimulated growth in vitro by adding them to the culture medium.

The effects of chemical substances on embryonic growth were studied by several investigators. In 1929 Allen and Corner established that injections of corpora lutea extracts into ovariectomized rabbits would support the development of rabbit ova through implantation. Burdick and Pincus (1935) showed that large injections of estrogen inhibited the growth of ova in utero, and in 1937 Pincus and Berkman found an increased concentration of ascorbic acid in the uteri of rabbits during the period of blastocyst growth. In 1933 Gregory and Goss reported finding a higher concentration of compounds containing sulfhydryl groups in developing embryos of giant strains of rabbits and chickens

than in corresponding smaller strains. These giant strains of rabbits also had a higher rate of cleavage during early embryogeny than did the smaller rabbit strains. The importance of sulfhydryl compounds for the cleavage of mammalian ova was further demonstrated by Pincus and Werthessen (1938) when they showed that simultaneous injections of glutathione and progesterone were effective in stimulating the growth of rabbit ova in utero.

In 1938 Pincus and Werthessen experimentally analyzed the results of these investigations. By experimenting on rabbit does ovariectomized twenty hours after copulation, they showed that injections of ascorbic acid inhibited ova growth, injections of either glutathione or progesterone alone would not stimulate growth, simultaneous injections of estrogen and progesterone inhibited ova growth, but simultaneous injections of glutathione and progesterone stimulated the growth of early embryos. From these results they advanced the theory that progesterone activity made glutathione available for the growth stimulation of ova, that excessive amounts of ascorbic acid reduced the effective concentration of glutathione by oxidizing it to an inactive state, and that estrogen activity inhibited progesterone activity and thus blocked the release of glutathione.

The in vitro studies following this hypothesis showed that the simultaneous addition of cysteine, similar in action but less effective than glutathione (Borsook et al., 1937; Barron and Singer, 1945), and ascorbic acid to

plasma clots did not induce the rabbit blastocysts to grow. It was also found that progesterone in plasma clots did not stimulate cleavage; however, it was not reported whether simultaneous additions of glutathione and progesterone were tested (Pincus and Werthessen, 1938). In 1940 Miller and Reimann reported that DL-methionine and L-cysteine did stimulate blastomere proliferation of rabbit eggs in vitro.

The results of these experiments indicated that the mechanism for the growth stimulation of early embryos cannot be explained completely on the basis of sex hormones; nevertheless, it was commonly believed that the female provided some morphogenetic hormones essential for embryonic growth. Although the uterus is the most efficacious position for embryonic growth and differentiation, it was known that the intrauterine environment was not essential for the growth of embryos, since embryos frequently implanted and developed in extrauterine positions. For example, Nicholas (1934) severed the lower oviducts in rats following copulation and two per cent of the embryos escaping into the abdominal cavity produced abdominal pregnancy. In these cases, however, the embryos were still under the influence of the maternal hormones which could reach the embryos via the circulatory system. Evidence that the maternal environment was wholly unessential for embryonic growth was presented by Nicholas in 1942. He obtained similar patterns of growth and differentiation from eggs and egg cylinders of the rat transplanted under the kidney capsule of either male or female host rats.

Further evidence was provided by Runner (1947) and Fawcett et al. (1947) when they showed that successful growth of mouse ova could occur in the anterior chamber of the mouse eye of both male and female hosts.

Recent advances in histochemical techniques have helped to identify the metabolites present in the uterine fluid, uterine endometrium, and the extraembryonic membranes. In 1939 Bloch showed that there was a substantial decrease in an osmiophilic, lipoid substance of the uterine epithelium during early pregnancy. She thought that the blastocysts were utilizing it, but Alden (1947) demonstrated that a high concentration of progesterone also resulted in the disappearance of the lipid. He believed that the natural increase in the progesterone concentration just prior to blastocyst implantation accounted for the disappearance of the lipid. Wislocki and Dempsey (1945) found an abundance of iron, alkaline phosphatase, and acid phosphatase in the endometrial glands of rodents during pregnancy. Wislocki et al. (1946) detected large quantities of glycogen in the decidua basalis and visceral yolk sac of rodents. They also demonstrated acid phosphatase activity in rodent placentae at term. In 1953 Dempsey found, with the aid of an electron microscope, that the cytoplasmic ultrastructure of the epithelium of the guinea pig visceral yolk sac was canalicular, which possibly facilitated the absorptive capacities of these cells.

It has been shown by Briones and Beatty (1954) that the uterine environment of one species will not support

growth and differentiation of the ova or early blastocysts of another species. Therefore, it seems highly unlikely that the simple addition of substances known to support cell growth in vivo or in vitro can provide a satisfactory medium for stimulating the very fastidious blastocyst to grow. A different experimental approach seems necessary. Perhaps an analysis of some of the metabolic activities of the blastocyst, uterine endometrium, and extraembryonic membranes would furnish new information helpful in understanding some of the unsolved problems in uterine-embryonic relationships.

Various techniques have been used by several investigators to obtain ova and early embryos from the oviducts and uteri of mammals (Nicholas and Rudnick, 1933; Lewis and Wright, 1935; Simpson and Williams, 1948; Hammond, 1949; Chang, 1950; Grobstein, 1950; and Beatty, 1951). Runner (1947) found that it was not necessary to maintain ova at body temperature since ova survived very well at room temperature during operations. Indeed, Chang (1947) found that fertilized rabbit ova best survived periods of storage at temperatures ranging from 10° C. to room temperature.

The length of time a mouse blastocyst will survive and metabolize normally in tissue culture is uncertain. Kirkham (1916) showed that implantation was delayed for as many as nine days in lactating mice. During this time the embryos remained in the blastocyst stage. These blastocysts carried on a maintenance metabolism but did not grow or differentiate. In 1946 Enders and Pearson reported the recovery of seventeen

blastocysts from the uterus of a mink during the period when the mink blastocysts were dormant prior to implantation. These blastocysts were then cultured in Tyrode's and Ringer's solutions. Their observations of the mink blastocysts indicated that they had survived in vitro for a period of five months. During this period, however, no growth occurred. Whitten (1956) reported culturing mouse morulae in vitro for two days and when the early blastulae arising from these cultures were transplanted under the mouse kidney capsule, they proceeded to grow much like blastulae of similar ages taken directly from mouse uteri and transplanted under the kidney capsule. From these experiments it can be concluded that growth is not an essential result of blastocyst metabolism in vitro. It may be that the metabolism of cultured ova is similar to the metabolism of blastocysts in a state of delayed implantation. Perhaps this type of metabolism is qualitatively different from that of rapidly growing blastocysts.

Crystallographic analyses showed that the bicarbonate content was very high in the fluids in the cavity of rabbit blastocysts (Lutwak-Mann and Lasher, 1954). Manometric studies made by Bell et al. (1928) indicated that the placenta had a higher rate of anaerobic respiration than the other fetal tissues. In 1952 Winnick demonstrated the utility of tissue culture methods in studying metabolic activities of embryonic tissues. With the use of radioactive isotopes, Gerarde et al. (1952) and Fisher et al. (1953) found

that the essential amino acids could not be synthesized from glucose in tissue culture, whereas many of the nonessential amino acids were readily synthesized. Gerarde et al. (1952) also found that the heart and lung tissues had different capacities to utilize glycine for protein synthesis. In 1954 Villee demonstrated with the aid of radioactively labeled tracers that different quantities of metabolites were produced by various human fetal tissues in vitro. Thus, differences in biochemical activity characterized different tissues in vitro. It may reasonably be assumed that these differences in vitro are a true reflection of differences present in vivo. Markert (1955, 1956) showed how studies on substrate utilization in vitro could be used to analyze some aspects of cell differentiation during embryogenesis.

In reviewing the literature it becomes evident that little is known about the metabolism of early mammalian embryos. It is equally apparent that the metabolic functions of the extraembryonic membranes have not been completely investigated. The objectives of this investigation are to identify various aspects of the metabolism of early mouse embryos and to contrast the metabolism of early embryos with that of later stages of development. The degree of nutritional dependency of early embryos upon nutrients of maternal origin may be inferred from such studies. An effort has also been made to determine what metabolic contributions are made by the extraembryonic membranes to the fetus and to relate various aspects of metabolism in the extraembryonic mem-

branes to their possible role of converting nutritional substances received from the mother into nutrients for the fetus.

MATERIALS AND METHODS

The mice, Mus musculus, used in this investigation were highly inbred C57BL/6 females and males raised in our laboratory. To supplement these, fifty breeding C57BL/6 females were obtained directly from the Roscoe B. Jackson Memorial Laboratory in Bar Harbor, Maine. The mice were kept in wooden boxes where they ate and drank ad libitum. The fertilized females, identified by the presence of a copulation plug, were separated from the males. Under natural diurnal lighting mice generally ovulate and copulate about midnight; therefore, the ages of the embryos are recorded from the average time of copulation. Fertilization most likely occurred within two hours after copulation (Runner and Palm, 1952, 1953).

The glassware used for tissue culturing was soaked first in alconox solution, washed and rinsed thoroughly with tap water, rinsed with distilled water, and finally rinsed with demineralized water (distilled water which had been run through a Bantam Demineralizer Cartridge manufactured by the Barnstead Still and Sterilizer Co., Boston 31, Mass.). The glassware was then air dried, wrapped in paper, and sterilized in the autoclave at a gauge pressure of 15 pounds per square inch for twenty minutes.

The metal forceps, needles, and scissors used for

tissue culturing were soaked in alconox solution, washed, and rinsed in the same manner as the glassware. However, these tools were sterilized in boiling demineralized water rather than in the autoclave. These instruments were used repeatedly throughout an operation and were kept sterile by replacing them in boiling water.

The radioactive substrates used in this study were uniformly labeled glucose and fructose and will be referred to as U-glucose-C-14 and U-fructose-C-14. The U-glucose-C-14 was obtained from Volk Radio-Chemical Company, 5412 No. Clark Street, Chicago 40, Illinois and had a specific activity of one millicurie per 8.7 milligrams of glucose. The U-fructose-C-14 was purchased from New England Nuclear Corporation, 575 Albany Street, Boston 18, Mass. and had a specific activity of one millicurie per 4.5 milligrams of fructose. The substrates were diluted with sterile distilled water or saline so that one-tenth of a millliliter of the diluted solution contained twenty microcuries of radioactivity. The purity of the radioactive substrates was checked by two dimensional paper chromatography and autoradiography as illustrated by Figure 1.

The physiological saline used for operating medium while the tissues were being dissected and cut into small tissue explants was Parker's 703 medium (see Table 1), which was modified by the deletion of glucose and antibiotics. This medium was also used in preparing the embryo extract and as the nutrient medium for the roller tube cultures.

TABLE 1

SYNTHETIC MEDIUM No. 703[a]

	Mgm. per 1000 ml.		Mgm. per 1000 ml.
Inorganic salts		Vitamins (continued)	
NaCl	6800.0	Folic acid	0.01
KCl	400.0	Choline	0.50
$CaCl_2$	200.0	Inositol	0.05
$MgSO_4.7H_2O$	200.0	p-Aminobenzoic acid	0.05
$NaH_2PO_4.H_2O$	140.0	Vitamin A	0.10
$NaHCO_3$	2200.0	Ascorbic acid	50.00
Fe, as $Fe(NO_3)_3$	0.1	Vitamin D	0.10
		Vitamin E	0.01
Amino acids		Vitamin K	0.01
l-Arginine	70.0		
l-Histidine	20.0	Lipid sources	
l-Lysine	70.0	Tween 80	5.0
l-Tyrosine	40.0	Cholesterol	0.2
dl-Tryptophane	20.0		
l-Cystine	20.0	Coenzymes	
dl-Methionine	30.0	Diphosphopyridine nucleotide (DPN)	0.78
dl-Serine	50.0	Triphosphopyridine nucleotide (TPN)	0.42
dl-Threonine	60.0	Coenzyme A	0.27
dl-Leucine	120.0		
dl-Isoleucine	40.0	Miscellaneous	
dl-Valine	50.0	Sodium acetate	50.0
dl-Glutamic acid	150.0	Glutathione	10.0
dl-Aspartic acid	60.0	l-Glutamine	100.0
dl-Alanine	50.0	Glucose	1000.0
l-Proline	40.0	Phenol red (pH indicator)	15.0
l-Hydroxyproline	10.0	Ethyl alcohol (vitamins A, D, and K, and cholesterol solvent)	16.0
dl-Phenylalanine	50.0		
Cysteine.HCl	260.0		
Glycine	50.0		
		Antibiotics	
Vitamins		Sodium penicillin G	1.0
Thiamine	0.01	Dihydrostreptomycin sulphate	100.0
Riboflavin	0.01	n-Butyl para-hydroxybenzoate	0.2
Pyridoxine	0.025		
Pyridoxal	0.025		
Niacin	0.025		
Niacinamide	0.025		
Pantothenate	0.01		
Biotin	0.01		

[a]Healy, G. M. et al. 1954 Nutrition of animal cells in tissue culture. IX. Synthetic medium No. 703. Canad. J. Biochem. Physiol. 32:327-337.

The plasma clots were made from nearly equal volumes of mouse blood plasma and mouse embryo extract. Plasma was obtained from blood drawn from hearts of four to ten day pregnant mice, which had been anesthetized with ether. A two milliliter hypodermic syringe with a twenty-five gauge needle was coated with a twenty milligrams percent solution of heparin. One-half milliliter of the heparin solution was drawn into the syringe. With the syringe held in a vertical position, the solution was worked over the inner surface by raising and lowering the plunger several times. The excess heparin solution was then removed by completely inserting the plunger into the syringe. Enough heparin remained in the needle to prevent blood clotting. The skin of the anesthetized female was removed as will be described later. A small fenestra was cut in the ventral portion of the thoracic cavity exposing the heart. The hypodermic needle was placed in a cavity of the beating ventricles and about one milliliter of blood was drawn into the syringe. The needle was removed from the syringe, the blood was transferred into a tube and centrifuged. The supernatant plasma was pipetted into paraffin coated tubes and stored in a refrigerator.

The embryo extract was made from eleven to thirteen day old embryos. After the extraembryonic membranes were removed, the embryos were homogenized in a tissue homogenizer and the homogenate was diluted with an equal volume of Parker's 703 medium. The diluted mixture was stored at -15° C. in a centrifuge tube for not longer than one week. The

cell debris was centrifuged down just before use and the clear supernatant, embryo extract, was used to clot the blood plasma.

The uteri of pregnant mice were removed in the following manner. The mouse was killed by cervical separation by placing the thumb and index finger on each side of the neck and squeezing quickly. It was then placed on its abdomen and an incision made in the skin on the dorsal surface. The skin was completely removed by pulling anteriorly and posteriorly from the dorsal incision. In this manner the microörganisms in the hair, which could easily contaminate the abdominal surface, were removed with the skin and the abdominal area was left sterile. The abdomen was opened by cutting anteriorly from near the vaginal opening towards the sternum. The reproductive organs were severed from their dorsal mesenteries and transferred into a small petri dish containing Parker's 703. The mesenteries and fat were removed with watchmaker's forceps because the lipoid droplets caused a turbidity in the operating medium.

The blastocysts were flushed from the uterine lumen in a manner similar to the method used by Chang (1950). After the oviducts were removed, the uteri were placed in a concave depression in a spot plate containing one milliliter of operating medium. About one-half milliliter of operating medium was drawn into a hypodermic syringe through a twenty-seven gauge needle. The needle was inserted into the cervical portion of the uterus and the medium was flushed through

the uterine lumen towards the oviducal end. The blastocysts were then located with a dissecting microscope and were transferred into another spot depression for washing and collecting. The maximum number of blastocysts was obtained from four day pregnant animals in this strain of mice. Before this time a number of blastocysts were still in the lower part of the oviducts, whereas many blastocysts were already implanted by four and one-half days after fertilization.

The blastocysts were cultured in plasma clots when U-glucose-C-14 was used as the substrate. They were transferred with a capillary pipette from the spot plate into a drop of blood plasma on a full size coverslip, 24 mm. by 60 mm. When the embryos had settled to the bottom of the plasma drop, an attempt was made to remove most of the operating medium added to the drop of plasma when the blastocysts were transferred. Then a drop of blood plasma was added to the culture, followed by a drop of Parker's 703 medium containing ten microcuries of U-glucose-C-14, and, finally, a drop of embryo extract was added to clot the plasma. The coverslip was sealed to a tissue culture slide with melted paraffin and the culture was placed in an incubator at 37° C.

In experiments on the utilization of fructose by the blastocysts a special culture chamber was prepared by drilling a small cylindrical pit, two millimeters in diameter and two millimeters deep, into one edge of the concavity of a depression slide. The manufacture of this vessel was done to

provide a container for culturing blastocysts in a small volume of medium which contained no glucose. It was believed that a small volume of fluid would be helpful for promoting the growth of blastocysts in much the same way that it was helpful for culturing isolated cells (Sanford et al., 1948). In these studies U-fructose-C-14 was dissolved in Earle's saline (Sanford et al., 1948) so that the final concentration of U-fructose-C-14 was one hundred milligrams percent; this medium was pipetted into the cylindrical pit containing the blastocysts.

The extraembryonic membranes used in this study were the visceral yolk sac, allantois, chorion frondosum, chorioallantoic placenta, and amnion. These structures were dissected from embryos ranging in age from nine and one-half to nineteen days. Uterine endometrium from mature virgins in estrus and from four and one-half day pregnant mice, and modified endometrium (decidua) from nine and one-half day pregnant mice were also used. Each of these tissues was cut into small tissue explants of about one cubic millimeter and washed in fresh medium to remove excess blood or tissue fragments before culturing.

The extraembryonic membranes and endometrium were cultured in roller tubes. The small tissue explants were pipetted into the tubes and evenly spaced along one wall. After the excess transferring medium was removed, a thin plasma clot was made in the area of the tissue explants to help affix them to the glass. One milliliter of Parker's 703 was

added and the roller tubes were incubated in a roller drum. The type of cellular outgrowth, changes in pH indicated by the phenol red in the medium, and the general appearance of the cultures were noted frequently. When a culture became well established and looked healthy a drop of Parker's 703 or distilled water containing ten microcuries of U-glucose-C-14 or U-fructose-C-14 was added.

After the tissues had metabolized for periods of time which were thought sufficient to produce measurable quantities of metabolites from the substrates, they were prepared for paper chromatographic analyses. Several preparative procedures were tried. It was found that the constituents of homogenized cell suspensions did not resolve well by paper chromatography. Some tissues were hydrolyzed with six normal hydrochloric acid in sealed vials in the autoclave for six hours at fifteen pounds gauge pressure per square inch. Acid hydrolysis destroyed all of the carbohydrates but it did completely hydrolyze the proteins into amino acids. Some cell suspensions were frozen and thawed with dry ice and subsequently digested with an equal volume of a three percent trypsin solution in the tissue culture incubator for twenty-four hours. With this procedure the proteins were incompletely digested by the trypsin and poor resolution was obtained on the paper chromatograms.

The following method, however, proved satisfactory and was used in analyzing the majority of the tissues. The plasma clot or the content of a roller tube culture was

transferred to a centrifuge tube. The tissue was alternately frozen and thawed several times with dry ice and warm water to rupture the cell membranes. One milliliter of ninety-five percent alcohol was added and the centrifuge tube was placed in a beaker of dry ice for an hour. The protein precipitate was centrifuged down and the clear supernatant fluid was removed. The precipitate was extracted once again with one milliliter of alcohol, the protein was centrifuged down, and the second supernatant fluid was added to the first.

The protein precipitate was air dried to remove the residual alcohol and then placed in a vial with one milliliter of six normal hydrochloric acid. The vial was sealed by flaming the tip and drawing it out with a pair of forceps. After hydrolysis for six hours in the autoclave at fifteen pounds pressure per square inch, the excess hydrochloric acid was removed by azeotropic distillation with water. Demineralized water was added to the hydrolysate in a centrifuge tube making a total volume of eight milliliters, which was evaporated down to about one milliliter in a water bath; whereupon, demineralized water was added again to bring the volume to eight milliliters and this was again evaporated. This process was repeated three times in all. A small jet of air was blown over the surface to aid the evaporation of the hydrochloric acid. The hydrolysate was finally concentrated to about three-tenths of a milliliter. An aliquot of the concentrated hydrolysate was spotted one inch from the bottom and left hand edge of Whatman No. 1 chromatography paper

cut fourteen by sixteen inches to fit in an x-ray casette. The spot was kept as small as possible and usually did not exceed one centimeter in diameter. An electric hair drier was used to evaporate the aliquot and to keep the spot small. An amino acid supplement, a solution of known amino acids, was usually added to the spot so that the quantity of each amino acid would be sufficiently large to produce a positive ninhydrin color reaction.

The alcoholic extract was concentrated by evaporating the alcohol with a current of air. The extract was not evaporated in a boiling water bath because some of the alcohol soluble organic extracts are volatile or easily destroyed by heat. If any precipitate formed while the alcoholic extract was being concentrated, it was removed by centrifugation and the clear supernatant fluid was spotted on a chromatogram and air dried. The precipitate was usually very small and was discarded. An organic acid supplement was added to the spot as a carrier and to increase the quantity of the acids so that a color reaction could be used to identify the metabolites.

The amino acids were resolved by two dimensional ascending chromatography. The irrigating solvents were phenol and lutidine (Berry et al., 1951). The phenol solvent for each chromatography jar contained one hundred and fifty grams of phenol (Mallinckrodt, Analytical Grade) saturated with an aqueous solution containing six and three-tenths percent sodium citrate and three and seven-tenths percent

potassium di-hydrogen phosphate. About thirty milliliters of this buffer were needed to saturate the phenol. The lutidine solvent contained ninety-seven and one-half milliliters of 2,6-lutidine and fifty-two and one-half milliliters of demineralized water. These irrigating solvents were allowed to saturate the atmosphere in the chromatography jars before the sheets were inserted.

The organic acids were also resolved by two dimensional paper chromatography. The first irrigating solvent contained one hundred and thirty milliliters of ether, thirty milliliters of glacial acetic acid, and ten milliliters of water for each chromatography jar (Denison and Phares, 1952). The second irrigating solvent was the 2,6-lutidine as described above.

The edges of a chromatogram were stapled together to form a cylinder before the chromatogram was placed in a chromatography jar with solvent. The chromatogram of the protein hydrolysate was placed in the autoclave and steamed without pressure for about ten minutes. This treatment uniformly saturated the fibers of the paper and the amino acids were resolved better. The chromatogram was then placed into a jar containing the phenol solvent for approximately thirty hours, at which time the solvent front had reached a height of fourteen inches. The chromatogram was removed and dried in the hood for a day or longer. It was then rotated ninety degrees and again stapled to form a cylinder. It was again steamed and then placed in the lutidine solvent. After about

twenty hours, when the solvent had migrated to a height of thirteen inches, the chromatogram was removed and dried for a day or more in the hood.

The chromatogram containing the organic acids was not steamed before chromatography because of the instability and volatility of some organic acids at elevated temperatures. The chromatogram was placed in the first solvent for twelve hours, at which time the solvent front had reached a height of fourteen inches. After drying for a day it was placed in lutidine for about twenty hours, removed and dried once again.

Each dry chromatogram was then placed in a Permacon casette (manufactured by General Electric, Milwaukee, Wisconsin) upon a sheet of No Screen Kodak Medical X-ray Film (manufactured by Kodak Corporation, Rochester, New York). More than one chromatogram and film could be placed in a casette by separating each set (chromatogram and film) from the other sets with a sufficient thickness of paper to prevent the penetration of the beta rays from carbon fourteen. After a sufficient time of exposure the film was removed and developed with Kodak developer and fixative. The darkened areas on the developed film locate the areas which were exposed to the beta ray emission of carbon fourteen.

After autoradiography the chromatograms were then developed to locate the positions of the various metabolites. The amino acids were identified by their specific color reactions with ninhydrin and also by their characteristic R_f values. About twenty milliliters of a two-tenths percent

solution of ninhydrin dissolved in water saturated butanol were sprayed on a chromatogram with an atomizer. The chromatogram was then placed in a warming oven at 90° C. for five minutes to promote a rapid ninhydrin color reaction with the amino acids (Berry and Cain, 1951). The organic acids were identified by their specific color reactions with ammoniacal silver, fluorescence, and characteristic R_f values. About twenty milliliters of a one-tenth normal ammoniacal silver solution were sprayed on a chromatogram and within four hours the different organic acids developed contrasting colors. The organic acids could be identified further by ultraviolet fluorescence of the ammoniacal organic acid complex (Buch et al., 1952).

The radioactive metabolites on the chromatograms were easily located with the aid of the autoradiogram since the darkened areas on the autoradiogram corresponded to the positions of radioactive metabolites on the chromatogram, as illustrated by Figures 11 and 12. An accurate determination of the radioactivity in each metabolite could not be made from the autoradiogram, even though the relative darkening on the autoradiogram varied directly with the amount of radioactivity in the metabolites. Therefore, the radioactivity in each metabolite was determined by counting. A geiger tube was firmly suspended one-sixteenth of an inch above a table top, a chromatogram was slipped under the geiger tube, the areas peripheral to the metabolite being counted were shielded with x-ray film, and the radioactivity of each metabolite

was assayed. The geiger tube had a 1.8 mg/cm^2 window and it was attached to a Raychronix pre-amplifier and counter (manufactured by Radioactive Products Inc., Detroit, Michigan).

The total radioactivity on different chromatograms was variable and a meaningful comparison of the radioactivity in metabolites on different chromatograms could not be made directly. However, a valid comparison could be made among the chromatograms of different tissues after converting the radioactivity measurements into relative radioactivity as compared to the radioactivity of alanine used as a standard.

RESULTS

The principal results of this investigation are those obtained from the utilization of labeled substrates by the cells of the blastocysts, extraembryonic membranes, and uterine endometrium. The patterns of cellular outgrowth produced by these tissues in vitro are also important in interpreting the results. Although these tissues were grown in a variety of media, the results reported here, with the exception of those for blastocysts, are restricted to those experiments in which the tissues were grown in roller tubes according to the techniques described previously. Thus, in vitro conditions were kept uniform for all the tissues except the blastocysts. Three tables will be found following this section summarizing the results of the in vitro growth and of U-glucose-C-14 and U-fructose-C-14 metabolism by these tissues. Autoradiograms of chromatograms, which illustrate the results obtained from some tissues, will be found in the appendix.

In vitro culture of blastocysts.-The varied attempts in the present investigation to culture mouse blastocysts deserve extended comment because many previous investigators have labored diligently without much success to grow blastocysts in vitro. Both culture media and various types of culture vessels were tested for their capacity to promote

the growth of blastocysts.

Initially, four-celled embryos obtained from oviducts of mice sixty hours after copulation were placed in approximately three-tenths of a milliliter of Parker's 703 medium in tissue culture depression slides. Such young embryos underwent no further cleavage and began to degenerate in two or three days. Likewise, blastocysts flushed from uteri of mice four and one-half days after copulation degenerated within two days under similar conditions of culture. The volume of air present in these culture chambers greatly exceeded that presumably available to blastocysts in vivo. To simulate conditions in utero, blastocysts were drawn into small capillary tubes containing Parker's 703 medium; the tubes were then sealed at each end with melted paraffin. In this anaerobic environment the blastocysts did not manifest outward signs of cellular disintegration for culture periods exceeding a month. However, when the capillary tubes were broken and the blastocysts liberated into fresh culture fluid, they exhibited great fragility. When transferred with pipettes the cells fell apart more easily than the cells of freshly recovered blastocysts.

Some blastocysts were cultured in aqueous humor obtained from adult mouse eyes, but they failed to grow in this natural medium. This result was somewhat surprising in view of the growth of blastocysts implanted into the anterior chamber of mouse eyes (Runner, 1947).

Mouse blastocysts underwent rapid disintegration

when they were cultured in Parker's 703 medium modified by the metabolic activity of pieces of uterine endometrium placed in the medium for twenty-four hours. Blastocysts cultured directly upon pieces of uterine endometrium or within excised uteri also failed to grow in vitro.

A chromatographic analysis was made of uterine fluid removed at the time of blastocyst implantation. This uterine fluid, as well as that from nonpregnant mice, contained several free amino acids in concentrations similar to those of common growth medium, such as Parker's 703. In an alcohol-soluble and heat-stable portion of the uterine fluid, a ninhydrin-positive substance possessing R_f values and a ninhydrin color reaction identical to taurine was present in large amounts. The paper chromatograms also showed that the concentrations of glutathione and cysteine were lower in the uterine fluid of pregnant mice.

Blastocysts were also cultured in fresh chicken plasma and chick embryo extract. Some blastocysts appeared to remain healthy in chicken plasma clots for periods of a month, indicating that the plasma clot was perhaps more nutritious than synthetic media or afforded better protection from hostile environmental influences.

In 1949 Chang reported that some heterologous sera had deleterious effects on the growth of fertilized rabbit ova. Therefore, mouse plasma and mouse embryo extract were used in later studies in preference to chicken plasma and chicken embryo extract. In general, the blastocysts survived

no better in mouse plasma cultures than in chicken plasma clots. However, it was believed, a priori, that mouse plasma should be a more favorable medium in which to study the normal metabolism of the fastidious mouse blastocysts. Experiments showed, however, that the metabolism of glucose by blastocysts in chicken plasma and in mouse plasma was similar.

In an attempt to determine more accurately the length of survival of mouse blastocysts in vitro, blastocysts of C57BL/6 mice were cultured for various periods of time in plasma clots before reimplantation into uteri of BALB/c mice at the appropriate stage of gestation. The results of these experiments are still inconclusive and will be reported at some later date.

Chang (1950) found that six day old rabbit blastocysts survived better in culture than four day old blastocysts. Likewise, in this investigation, older mouse blastocysts survived in vitro culture better than younger stages of development. Preferably, the blastocysts should be flushed from the uteri just prior to implantation. By this time the zonae pellucidae have been dissolved, the blastocysts have become elongated, the inner cell masses have become conspicuously larger, and there are a larger number of cells to metabolize available substrates. However, blastocysts were obtained from several mice for each experiment and the ova were not fertilized at precisely the same time in all these mice. Some variation in embryonic age was therefore inevit-

able. Blastocysts in some mice had implanted while the zonae pellucidae remained intact on the blastocysts from other mice. Blastocysts from any one mouse lie freely in the uterine lumen for about six to ten hours and the time of fertilization of different mice probably also varied as much as six hours. The maximum number of blastocysts could be flushed from the uteri at four days after copulation in this strain of mice. At this time the zonae pellucidae of the more advanced blastocysts were almost completely dissolved.

Growth and metabolism of mouse blastocysts in vitro.- About twenty-five percent of the zonae pellucidae dissolved during the period of incubation in plasma clots. Hall (1935) had previously found that the zonae pellucidae could be dissolved by slightly acid media in hanging drop cultures. It seems probable that the progressive dissolution of the zonae pellucidae in these experiments resulted from an increase in acidity in the plasma clots as a consequence of cellular metabolism. Thus the dissolution of the zonae pellucidae may be considered an indicator of metabolic activity in the cultures even though growth was not observed.

The cell membranes in the trophoblastic regions of some blastocysts were mapped immediately after culturing. No cell division was observed over a period of twenty-four hours, although two cell divisions normally occur during this time in utero. In a few blastocysts the inner cell masses became enlarged and protruded into the trophoblastic cavity, forming an egg cylinder anlage. Thus, some cell division was

possibly occurring in the inner cell mass during culture. Stained whole mounts of slightly squashed preparations of cultured blastocysts revealed normal nuclei without evidence of pyknosis during the first few days of culture.

Mouse blastocysts in plasma clot cultures developed blebs of trophoblastic origin during the first few days. These blebs contained lipoid droplets and a watery fluid less viscous than the cytoplasm of freshly recovered blastocysts. Initially, the deuteroplasm of the trophoblastic cells was apparently metabolized, as is commonly true for these embryonic cells (Lewis and Wright, 1935). Subsequently, the normal semipermeability of the cell membranes disappeared and osmosis caused the cells to swell and the blebs to form.

The analyses of blastocyst cultures after periods of metabolism in the presence of radioactive glucose or fructose showed that the blastocysts were able to absorb these radioactive substrates and convert them into other metabolites. The metabolites, which were derived at least in part from the glucose and fructose metabolized by the cells of the mouse blastocyst, were lactate, as illustrated by Figure 2, alanine, serine, and glycine (Table 3). In four cultures containing twenty-four, twenty-eight, forty-five, and fifty-nine blastocysts respectively, the quantities of lactate formed by the blastocysts from U-glucose-C-14 were proportional to the number of blastocysts in each culture. Many amino acids frequently synthesized from metabolized sugars, such as glutamate, aspartate, proline, histidine, and cysteine, were

not formed in detectable amounts from the radioactive glucose and fructose made available to blastocysts in vitro.

Growth and metabolism of uterine endometrium in vitro.- Pieces of endometrium cultured from females in estrus and at four and one-half days of gestation had similar growth patterns in vitro. Numerous sheets of epithelial cells and a few macrophages arose from explants of endometrium, but fibroblasts were never observed. The macrophages appeared early in culture, while the epithelial cells became more numerous with prolonged periods of culture (Table 2).

Metabolic patterns for uterine endometrium cultures at estrus and at the time of implantation were qualitatively alike (Table 3). Pyruvate and lactate on the chromatograms of the alcoholic extracts of uterine endometrium following metabolism in U-glucose-C-14 contained radioactive carbon in measurable amounts. The autoradiograms of the chromatograms of hydrolyzed proteins showed that glutamate, aspartate, alanine, serine, glycine, proline, and an unidentified ninhydrin-positive substance were also derived from glucose and fructose metabolized by the pieces of endometrium at both phases. The unidentified ninhydrin-positive spot in the hydrolysate had R_f values of approximately .15 and .40 in the phenol and lutidine solvents respectively, as illustrated by Figure 3. Table 4 shows that the relative quantities of serine and glycine derived from U-fructose-C-14 are larger than those from U-glucose-C-14, while the relative amounts of the other amino acids change only slightly.

Growth and metabolism of decidua in vitro.-Explants of decidua capsularis and decidua basalis taken from females at nine and one-half days of gestation grew similarly in culture. A few fibroblasts arose within the first twenty-four hours, but they changed to macrophage-like cells within a few days. Other macrophages, apparently arising from blood in the tissue explants, were also observed in cultures of decidua. Relatively little cellular outgrowth was obtained in these cultures in view of the volume of tissue present in the pieces of explanted decidua (Table 2).

Although growth was not abundant, both the decidua basalis and decidua capsularis metabolized very rapidly during the first few days of culture, but the metabolic rate gradually diminished thereafter. The identified substances derived from the radioactive glucose and fructose metabolized by the decidual cells were lactate, alanine, serine, glycine, glutamate, proline, aspartate, and cysteine (Table 3). The amino acids derived from U-glucose-C-14 by the decidua capsularis and from U-fructose-C-14 by the decidua basalis removed from the uterus nine and one-half days after copulation are illustrated by Figures 5 and 6 respectively. Table 4 again shows that the relative quantities of serine and glycine synthesized from radioactive fructose are substantially larger than from radioactive glucose. It can also be seen from the table that the relative amounts of glutamate and aspartate are also increased during fructose metabolism by the decidua.

Growth and metabolism of placenta in vitro.-The cellular outgrowth produced by placental explants varied at different stages of development. As indicated previously, the decidua basalis, a precursor to the placenta, at nine and one-half days of gestation produced little cellular outgrowth in vitro. However, small bits of placenta removed during the twelfth through the fourteenth days of gestation developed a large number of giant size fibroblasts during the first few days of culture. These giant fibroblasts subsequently retracted their elongated cytoplasmic processes and became rounded. Pieces of placenta cultured from animals nearing parturition failed to produce these giant size fibroblasts. A few macrophages, probably arising from blood in the tissue explants, were seen in these cultures; however, the cellular outgrowth was very sparse for the quantity of tissue present in these cultures (Table 2).

A rapid increase in acidity, as shown by the changing color of phenol red in the medium, indicated that the placenta from all stages of development metabolized very rapidly. The in vitro metabolism of glucose and fructose was qualitatively the same at different periods of gestation (Table 3). Autoradiograms of the chromatographed alcoholic extracts showed that a large quantity of radioactive lactate and a smaller amount of radioactive pyruvate was present in the cultures after a period of metabolism. Autoradiograms of the chromatographed protein-hydrolysates revealed that alanine, glycine, serine, glutamate, aspartate, proline,

cysteine, and an unidentified ninhydrin-positive substance were also derived at least in part from the radioactive glucose and fructose metabolized by the placenta. The unidentified ninhydrin-positive substance had the characteristics mentioned previously. The autoradiograms also showed that more radioactive lactate was present in placenta cultures metabolizing U-glucose-C-14 than in cultures metabolizing U-fructose-C-14. However, it can be seen from Table 4 that the relative amounts of serine, glycine, glutamate, and aspartate synthesized by the placenta from radioactive fructose are larger than from radioactive glucose. It is also apparent from the table that the pattern of metabolism is changing throughout gestation. The comparative metabolisms of the rapidly growing placenta utilizing U-glucose-C-14 and U-fructose-C-14 are illustrated by Figures 7 and 8 respectively.

Growth and metabolism of the allantois in vitro.- The allantoic stalk explanted from nine and one-half and ten and one-half day embryos developed many fibroblasts in culture which later metamorphosed into macrophage-like cells. Cells of the allantois did not divide rapidly in vitro; however, they apparently remained healthy for extended periods of culture (Table 2).

The autoradiograms show that an appreciable amount of lactate and lesser amounts of alanine, serine, glycine, glutamate, aspartate, and proline were derived from the labeled glucose and fructose metabolized by the cells of the

allantois (Table 3). It was also found that the allantois metabolized glucose more rapidly than fructose, such that the quantity of amino acids derived from fructose was much smaller than from glucose. Therefore, it was not possible to make a valid quantitative comparison between glucose and fructose metabolism for amino acid synthesis as was done for many other tissues.

Growth and metabolism of chorion frondosum in vitro.- Tissue explants of chorion frondosum removed from ten and one-half day embryos produced a few fibroblasts during early culture; however, numerous clusters of epithelial cells began to appear after the first week. A few macrophages were also observed which probably arose from contaminating cells of the decidua basalis adhering to the fronds when the chorion frondosum was separated from the decidua (Table 2).

The metabolism of the chorion frondosum was similar to that of the allantois. The identified metabolites derived from glucose and fructose were lactate, alanine, serine, glycine, aspartate, glutamate, and proline (Table 3). Again the quantity of amino acids derived from fructose was very small and a valid comparison between glucose and fructose metabolism in the chorion frondosum could not be made.

Growth and metabolism of amnion in vitro.-Pieces of amnion taken from embryos at various intervals from the ninth day of gestation until just prior to parturition grew similarly in vitro. Sparse outgrowths of fibroblasts were observed shortly after culturing and these cells soon modi-

fied into macrophage-like cells. These macrophage-like cells further metamorphosed into isolated cells which looked much like epithelial cells; however, sheets of epithelial cells did not develop (Table 2).

The amnion apparently had different capacities to synthesize metabolites from glucose and fructose at different stages of development. Amnion from nine and one-half and ten and one-half day embryos produced substantial quantities of lactate, alanine, serine, and glycine, only trace amounts of glutamate and aspartate, but no proline from U-glucose-C-14. It was found that explants from thirteen and one-half day and older embryos produced proportionately larger quantities of glutamate and aspartate, and also detectable amounts of proline (Table 4). Figure 9 illustrates the synthesis of radioactively labeled lactate from U-fructose-C-14 by fourteen and one-half day old amnion.

Growth and metabolism of visceral yolk sac in vitro.- The visceral yolk sac in vitro produced different types of cellular outgrowth at various stages of embryonic development. The visceral yolk sac can be separated easily from the other tissues beginning at nine and one-half days of gestation. Cultures of the visceral yolk sac at this stage of development produced numerous fibroblasts during the first few days of incubation. However, sheets of epithelial cells were the only form of growth observed after three weeks of culture. Only a few macrophages were seen in cultures of visceral yolk sac from these young embryos.

Explants of visceral yolk sac removed from thirteen and fourteen day old embryos initially developed a moderate number of fibroblasts which later modified into macrophage-like cells. These cells accounted for only a small percentage of the total number of macrophages in these cultures. A larger percentage of the cells in the outgrowth from explants of visceral yolk sac at this stage of development were true macrophages. Another group of cells possessed characteristics of both fibroblasts and macrophages. They had long cytoplasmic processes like fibroblasts, but were also highly amoeboid, like macrophages. Usually only one or two cytoplasmic extensions were present on these intermediate type cells, whereas typical fibroblasts generally have a larger number of cytoplasmic processes giving them a triangular or stellate shape. Clusters of epithelial cells began to appear about the third day of culture and became more conspicuous after extended periods of incubation.

Tissue explants of visceral yolk sacs removed from embryos just prior to parturition developed fewer fibroblasts than explants from younger stages of development. The percentage of macrophages in these cultures was also reduced but the number of epithelial cells was larger (Table 2).

Although the patterns of cellular outgrowth in the cultures of visceral yolk sac at successive stages of development were different in many respects, the metabolic activities were qualitatively alike (Table 3). The metabolic rate of the visceral yolk sac was very rapid, particularly

after the twelfth day of gestation. During in vitro incubation the cells of the visceral yolk sac converted the labeled sugars into lactate, succinate, pyruvate, glutamate, proline, aspartate, histidine, cysteine, serine, glycine, alanine, and an unidentified ninhydrin-positive substance. Figure 10 illustrates the synthesis of lactate and succinate from radioactive glucose by nine and one-half day yolk sac cells. Figure 11 is an autoradiogram of a chromatogram (Figure 12) of the protein hydrolysate of nine and one-half day yolk sac following metabolism in U-glucose-C-14. It can be seen from Figures 13 and 14 that the labeling patterns of amino acids from radioactive glucose and fructose by thirteen and one-half and fourteen and one-half day old yolk sac cells, respectively, are qualitatively alike. Figure 4 shows that the labeling of amino acids from radioactive fructose by yolk sac cells just prior to parturition is qualitatively much like that of younger yolk sac cells. However, in the photographic reproduction of autoradiograms much of the various degrees of darkening present on the autoradiograms is lost; therefore, by consulting Table 4 again, one finds that the relative amounts of amino acids derived from labeled fructose and glucose are quantitatively quite different. These differences and the progressive changes in amino acid synthesis during gestation will be discussed later.

TABLE 2

CELLULAR OUTGROWTH OBTAINED FROM TISSUE EXPLANTS

Tissues	Postovulatory Age of Tissue	Rate of Metabolism	Fibroblasts	Macrophages	Epithelia
Blastocyst	4	low	-[a]	-	-
Uterine endometrium	0	high	-	1[b]	3[d]
Uterine endometrium	4½	high	-	1	3
Decidua	9½	high	1	1	1
Placenta	13½	high	3	2[c]	1
Placenta	19	high	-	1	1
Chorion frondosum	10½	low	1	1	2
Allantois	10½	low	2	1	-
Amnion	10½	low	1	1	-
Amnion	13½	moderate	1	1	-
Amnion	19	moderate	1	1	-
Visceral yolk sac	9½	moderate	2	1	3
Visceral yolk sac	10½	moderate	2	2	3
Visceral yolk sac	13½	high	2	3	2
Visceral yolk sac	19	high	1	2	3

[a]Absent.

[b]Few.

[c]Moderate.

[d]Abundant.

TABLE 3

LABELING OF AMINO ACIDS FROM RADIOACTIVE CARBOHYDRATES (QUALITATIVE RESULTS)

Products	Blastocyst		Uterine Endometrium				Decidua		Placenta	
	4 p.o.[a]		Estrus		4½ p.o.		9½ p.o.		13½-19 p.o.	
	G[b]	F[c]	G	F	G	F	G	F	G	F
Alanine	+	+	+	+	+	+	+	+	+	+
Serine	+	+	+	+	+	+	+	+	+	+
Glycine	+	+	+	+	+	+	+	+	+	+
Glutamate	-	-	+	+	+	+	+	+	+	+
Aspartate	-	-	+	+	+	+	+	+	+	+
Proline	-	-	+	+	+	+	+	+	+	+
Histidine	-	-	+	+	+	+	-	-	-	-
Cysteine	-	-	-	-	-	-	+	+	+	+

TABLE 3-Continued

Products	Amnion		Chorion Frondosum		Allantois		Visceral Yolk Sac	
	10½-19 p.o.[a]		10½ p.o.		10½ p.o.		9½-19 p.o.	
	G[b]	F[c]	G	F	G	F	G	F
Alanine	+	+	+	+	+	+	+	+
Serine	+	+	+	+	+	+	+	+
Glycine	+	+	+	+	+	+	+	+
Glutamate	+	+	+	+	+	+	+	+
Aspartate	+	+	+	+	+	+	+	+
Proline	±	±	+	-	+	-	+	+
Histidine	-	-	-	-	-	-	+	+
Cysteine	-	-	-	-	-	-	+	+

[a]Postovulatory age of tissue in days.

[b]U-glucose-C-14 substrate.

[c]U-fructose-C-14 substrate.

TABLE 4

LABELING OF AMINO ACIDS FROM RADIOACTIVE CARBOHYDRATES (QUANTITATIVE RESULTS)

Products	Uterine Endometrium				Decidua Capsularis	Decidua Basalis	
	Estrus		4½ p.o.[a]		9½ p.o.	9½ p.o.	
	12 i.[b]	4 i.	8 i.	4 i.	10 i.	10 i.	6 i.
	G[c]	F[d]	G	F	G	G	F
Alanine	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Serine	.38	1.37	.78	1.26	.09	.56	9.47
Glycine	.51	1.36	.50	.58	.22	.37	2.37
Glutamate	1.63	2.04	1.61	1.78	.60	1.13	5.71
Aspartate	.75	.83	1.00	1.09	.42	.54	2.69
Proline	.09	.05	.05	.05	.02	.06	.49
Histidine	.03	.02	.05	.02	.01	.01	.01
Cysteine	.02	.01	.01	.01	.02	.04	.38

[a]Postovulatory age of tissue in days at the time of explantation.

[b]Days of incubation.

[c]U-glucose-C-14 substrate.

[d]U-fructose-C-14 substrate.

Note:

Each column represents the results from one experiment and, although representative, it is not an average derived from several experiments. The numbers represent the relative radioactivity in each metabolite on a chromatogram following the ninhydrin reaction as compared to the radioactivity of alanine on the same chromatogram used as a standard.

TABLE 4-Continued

Products	Placenta				Amnion		
	13½ p.o.[a]		18½ p.o.		10½ p.o.	13½ p.o.	14½ p.o.
	7 i.[b]	7 i.	7 i.	7 i.	25 i.	23 i.	13 i.
	G[c]	F[d]	G	F	G	G	F
Alanine	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Serine	.76	5.01	.08	.17	.13	.30	6.75
Glycine	.25	.85	.10	.08	.15	.14	1.78
Glutamate	2.20	2.84	1.46	1.53	.06	.68	4.15
Aspartate	1.73	1.80	.47	.88	.05	.15	.67
Proline	.17	.08	.06	.05	.02	.01	.01
Histidine	.01	.01	.01	.02	...	...	...
Cysteine	.01	.08	.02	.03	...	...	...

a,b,c,d Ibid.

TABLE 4-Continued

Products	Amnion		Visceral Yolk Sac				
	19 p.o.[a]		10½ p.o.	13½ p.o.		18½ p.o.	
	18 i.[b]	14 i.	11 i.	7 i.	7 i.	7 i.	7 i.
	G[c]	F[d]	G	G	F	G	F
Alanine	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Serine	1.20	4.91	.68	.86	3.25	1.03	6.08
Glycine	.65	1.02	.55	.58	2.47	.60	2.04
Glutamate	2.11	1.50	1.22	1.14	1.15	.99	2.34
Aspartate	.73	.41	.96	.80	.83	.60	1.27
Proline	.11	.01	.01	.06	.04	.01	.04
Histidine	...	...	.01	.02	.05	.01	.03
Cysteine	...	...	.01	.04	.04	.01	.05

a,b,c,d Ibid.

DISCUSSION

The nature of the factors promoting blastocyst growth.- Although the uterus provides the optimal environment for the embryo, Nicholas (1942) and Runner (1947) demonstrated that the factors promoting growth of young embryos are not specifically uterine products. In this investigation it was found that pieces of uterine endometrium or whole uteri containing embryos cultured in vitro completely failed to provide the young embryos with the substances essential for morphogenesis. From these results it is possible to hypothesize that one of two possibilities exists. First, the metabolism of uterine endometrium in vitro may differ from normal uterine metabolism; or, second, the uterine endometrium per se does not normally synthesize the growth promoting factors or provide the specific environment necessary for promoting the growth of young embryos. Although the first possibility has not been disproven, it seems probable that the second is more correct since blastocysts will implant and develop in extrauterine positions. This implies, therefore, that the growth-promoting substances or regulating factors arise at least in part from extrauterine tissues. However, if the morphogenetic substances are transported to the uterus via the circulatory system, the blood of pregnant mice at the appropriate sexual phase should contain them. Since cultures

made in the blood plasma from pregnant mice were not superior to cultures made in blood plasma from nonpregnant mice, either the concentration of the growth-promoting substances in the blood may be below the threshold level or the substances may be very unstable.

If the first possibility is true, the areas supporting embryonic growth must effectively concentrate the substances sufficiently to stimulate cell division. Since the histology is very different in these sites, namely, in the uterus, in the anterior chamber of the eye, under the kidney capsule, in the omental bursa, and on the outer surfaces of the oviduct and uterus, it seems reasonable to rule out the possibility that each is a target area or possesses the same properties for concentrating the morphogenetic substances. On the other hand, unless an implanted embryo stimulates the development of an adequate vascular supply at the site of implantation, it fails to grow and differentiate. Therefore, it seems probable that the embryonic, growth-promoting factors are present in the blood but are very labile substances. Further evidence for the instability of the morphogenetic substances also arises from the discovery that the aqueous humor from the eyes of adult mice did not stimulate the growth of blastocysts in vitro.

It may also be deduced that the stimulatory substances are necessarily of small molecular size, since they pass the blood-brain barrier and reach the anterior chamber of the eye. Although Parker's 703 medium contains the

vitamins, coenzymes, amino acids, inorganic salts, and glucose essential for promoting the growth of several differentiated cells in vitro, these substances of small molecular size are insufficient for stimulating the growth of the less differentiated embryonic cells.

The possibility that the embryo receives some special morphogenetic substances as well as ordinary nutrients from the blood during early ectopic pregnancy is strengthened by the fact that the nutrients of the coelomic fluids alone can support the growth of large volumes of ascites cells. The embryonic morphogenetic substances are doubtless not unique substances in the blood functioning only to promote embryonic growth since they occur in the blood of both male and female hosts; therefore, these substances must also have other roles in the body. Since fresh blood plasma fails to stimulate blastocyst growth, it is also possible that the composition of the medium essential for growth of blastocysts is maintained in the body by a dynamic steady state and that these very precise conditions existing in vivo have not been duplicated as yet in vitro.

Although there is no experimental evidence available, it can be deduced from what is generally known that the oxygen tension is very low in the sites where early embryos will successfully implant. The observation that the blastocysts maintained their integrity better and did not exhibit autocatalysis in small capillary tubes under anaerobic conditions is further evidence that aerobic environments are

injurious to the blastocysts. The autocatalytic activity of early embryos in aerobic environments suggests that the regulation of the oxidation-reduction potential may be an important factor in maintaining a favorable environment for the growth of early mammalian embryos.

The presence of large amounts of taurine in the uterine fluid may be further evidence that the oxidation-reduction potential is an important factor for blastocyst growth. Although the function of taurine in biological systems is unknown, it is unlikely that taurine per se has any role in stimulating blastocyst growth. The abundance of taurine in the uterine fluid does suggest, however, that it may be a component or a derivative of a component present in the stimulating substances. Taurine may arise from cysteine by oxidation of the sulfhydryl group to sulfate by an unknown method and decarboxylation of the acid group by cysteic acid decarboxylase activity (Baldwin, 1952), or taurine may also be formed by the decarboxylation of cysteine sulfinic acid (Awapara and Wingo, 1953). Since one of the functions of cysteine in biological systems is regulating the oxidation-reduction potential, the taurine may have arisen from the cysteine used for this purpose within the uterine fluid.

Previous attempts to stabilize the oxidation-reduction potential in vitro by the addition of glutathione (Pincus and Werthessen, 1938) or methionine and cysteine (Miller and Reimann, 1940) were not very rewarding. Attempts to promote the growth of mouse blastocysts in vitro by the

addition of glutathione and progesterone were also unsuccessful. However, in view of the more recent findings, the role of the oxidation-reduction potential for promoting the growth of early mammalian embryos might profitably be investigated further.

Blastocyst metabolism.-The metabolism of blastocysts studied in this investigation was not the metabolism of rapidly growing embryos. As much radioactivity was found in the free amino acids obtained in the alcoholic extracts as in the bound amino acids obtained from the protein hydrolysates. In cultures of rapidly growing tissues, more labeling was found in the amino acids of the protein hydrolysate than in the free amino acids. Thus, metabolic equilibration has possibly occurred among the radioisotopically labeled compounds in these cultures. It is unknown how long mouse blastocysts in vitro will metabolize sufficiently to retain their capacity to grow again when placed in the proper environment. The culture of blastocysts in vitro and subsequent reimplantation into the uterus of another genotype will provide this information, which will also be very helpful in understanding more about the metabolism of mouse blastocysts.

As well as could be determined with the techniques used in this study, the mouse blastocysts in vitro respired anaerobically. This conclusion is based upon the facts that lactate was the principal end product of carbohydrate metabolism and that the amino acids generally arising in part by transamination of the alpha-keto acids of the citric acid

cycle, which is more active aerobically, were not synthesized in detectable quantities from the radioactive sugars. Since the blastocysts cannot synthesize many of the amino acids which are nonessential amino acids for more differentiated cells, a very important role of the uterine endometrium must be to supply directly for the blastocysts those amino acids which are essential constituents for all the proteins synthesized by the blastocysts for both cell maintenance and cell multiplication. The chromatographic analysis of the constituents in the uterine fluid showed that this role is carried out by the uterine endometrium since the uterine fluid contains amino acids in concentrations similar to those found in common growth media such as Parker's 703.

Uterine endometrium.-Other indirect evidence suggests that the metabolism of uterine endometrium supplies much of the embryotrophe for the blastocysts lying free in the uterine lumen; however, as mentioned previously, it probably does not supply the morphogenetic substances. Osmiophilic substances present in the epithelial cells of the endometrium are apparently the same osmiophilic substances found in the trophoblastic cells of the blastocysts (Bloch, 1939; Alden, 1947). In 1902 Jenkinson reported that the trophoblastic cells are able to engulf red blood cells present in the uterine lumen. Although the enzymology of the uterine epithelium has not been completely investigated, it has been shown thus far that carbonic anhydrase (Lutwak-Mann and

Lasher, 1954), alkaline phosphatase and glycogen (Wislocki et al., 1946), and succinic dehydrogenase (Padykula, 1953) are present there. A more complete investigation of the enzymes present in the endometrium would doubtless be very helpful in understanding more about the role of the endometrium in supplying nutrients for the developing embryo.

In this investigation it was found that the uterine endometrium apparently undergoes both anaerobic and aerobic metabolism in culture. The large quantity of lactate synthesized by the endometrium suggests that an appreciable amount of its metabolism proceeds anaerobically while the production of large quantities of aspartate, glutamate, and proline arising in part as transaminated products of the citric acid cycle indicates that the endometrium respires aerobically as well.

The formation of a large number of amino acids from labeled carbohydrates by the metabolizing endometrium indicates that the amino acids found in the uterine fluids quite probably were synthesized by the uterine epithelium rather than in more distant tissues and simply secreted into the uterine lumen. To verify this, the quantity of radioactively labeled, free amino acids synthesized by the endometrial cells and secreted into the medium during culture should be assayed and compared with the quantity of radioactively labeled, protein-bound amino acids within the cells. It is possible that all of the amino acids synthesized by the endometrium may have been incorporated into proteins directly;

however, on the basis of studies on other tissues assayed in this manner it is more probable that a large quantity of the amino acids synthesized by these metabolizing cells was secreted into the medium as well (Markert, 1956).

The uterine environment is different at the time of implantation since blastocysts transplanted into uteri at an improper stage of gestation will not develop. It can be seen from Table 4 that the pattern of carbohydrate metabolism of the uterine endometrium does not change much from the time of estrus until blastocyst implantation and it is not evident from these metabolic studies what these differences are.

Decidua.-Creighton in 1878 showed that the decidua arises from the undifferentiated perivascular mesenchymal cells, becomes highly perforated with blood lacunae, and, under the influence of a favorable endocrine situation, enlarges and accumulates large stores of glycogen and lipoid materials. By the ninth day of gestation the decidua composes most of the tissue within the uterus and completely encapsulates each growing embryo. The decidua forms a disjunctional zone for the placenta and supplies the food for the developing embryo. As pregnancy progresses beyond the tenth day, the quantity of glycogen in the decidua gradually diminishes. In contrast, the visceral yolk sac lying in close proximity to the decidua increases in glycogen content reaching a maximum concentration in the visceral yolk sac about the fifteenth day of gestation. About the same time the decidua capsularis is disintegrating and the glycogen in the

yolk sac is presumably absorbed from the disintegrating decidua (Wislocki et al., 1946).

Since the decidua arises from the uterine mucosa it might be expected that its pattern of metabolism would closely resemble the metabolism of the uterine endometrium. The decidua encapsulates the embryo and can readily provide large quantities of nutrients by diffusion. Metabolically, therefore, the decidua and uterine mucosa should function similarly in providing nutrients for the developing embryo. However, Table 4 shows that the pattern of metabolism in the decidua is different from the metabolism of the uterine endometrium. A close inspection of the data shows that the major differences between endometrial and decidual metabolism are that the quantity of serine derived from fructose is exaggerated, whereas the levels of amino acids relative to alanine are lowered with glucose. From the techniques used it is not possible to state conclusively that either the alanine production was greatly increased with glucose metabolism or the production of the other amino acids was diminished; however, the data seem to favor the former possibility.

The reduced synthesis of lactate may result in a reduction of alanine derived from fructose metabolism since there would be less pyruvate available for alanine formation. However, this still does not explain the reasons for a preferential synthesis of serine and, usually, glycine during fructose metabolism.

In addition to providing glycogen for the embryo, the in vitro metabolism indicates that the decidua may provide several amino acids for the extraembryonic membranes and the developing embryo. The availability of glycogen for embryonic metabolism is of considerable importance for the production of energy for developing embryos and will be discussed more extensively in a later section.

Placenta.-The maternal portion of the discoidal placenta arises directly from the decidua basalis. The fetal chorioallantoic vessels invade the decidua basalis and bathe in the blood lacunae connected with the maternal uterine blood vessels. The principal roles of the placenta are the absorption of nutrients from the maternal blood lacunae for the fetus and the removal of waste products of embryonic metabolism.

Since the placenta arises from the decidua basalis, it might be expected that the pattern of metabolism in the placenta would be similar to the metabolism of its precursor tissue, the decidua basalis. A close inspection of the data shows that one major difference is apparent between the metabolisms of the thirteen and one-half day old placenta and the decidua basalis removed at nine and one-half days after copulation. This difference is the relative decrease in alanine formed from glucose by the placenta.

The metabolism in the placenta undergoes a change just prior to parturition. This change, principally in glycine and serine synthesis, possibly is indicative of the onset

of senescent metabolism in this tissue.

Although the placenta is in an environment conceivably conducive to aerobic respiration, it produces a large concentration of lactate in tissue culture. Studies made by Bell et al. (1928) demonstrate that the placenta respires both aerobically and anaerobically. The results of in vitro metabolism in this investigation show that the placenta could convert large amounts of the glucose absorbed from the maternal blood stream into amino acids which may then be made available to the fetus; however, there is little evidence that this actually occurs in the mammalian placenta. Proteases have been found in all placentae examined, but no one has shown that they are involved in the hydrolysis or synthesis of proteins for the fetus. It has been shown, however, that the placenta concentrates by four to six fold some of the amino acids absorbed from the maternal blood stream (Christensen and Streicher, 1948).

Allantois.-The allantois arises from the posterior ventral region of the embryo at nine and one-half to ten and one-half days of development. It is a stalk of pure mesodermal cells since the endodermal component lining the inner surface of the allantois does not evaginate from the cloacal area of the hind gut in the mouse. The allantois is the precursor of the umbilical cord which supports the umbilical blood vessels traversing the space between the fetus and placenta. This important morphological role of the allantois is fairly well understood and it has been shown that its

development is controlled by a specific dominant gene. In homozygous dominant brachyury mice (T/T), the allantois fails to grow and the animals die in utero because the placental circulation, necessary for the further development of the embryo, is not established (Grüneberg, 1952). The metabolic deficiency associated with this gene has not been investigated and the techniques used in this investigation could be applied to the study of this problem.

A priori, the allantois might be thought to respire anaerobically in vitro, since at the time of explantation it does not have a vascular supply. It does, nevertheless, demonstrate a fair capacity for aerobic respiration in vitro. This aerobic capacity is possibly in part due to the position occupied by the allantois in the embryo. The allantois buds off the hind gut and grows into the exocoelom formed by the visceral walls of the inverted yolk sac. Brambell (1954) demonstrated that the fluid in the exocoelom contains many substances entering by diffusion through the visceral yolk sac. It is conceivable that sufficient oxygen also diffuses into this fluid to provide for aerobic respiration in vivo.

Chorion frondosum.-The chorion frondosum is composed of the splanchnic mesoderm and ectoderm from the trophoblast. It is invaded by the allantoic blood vessels about the tenth or eleventh day of gestation; together they form the fetal portion of the placenta. The fronds on the chorion frondosum delineate the chorion frondosum from the peripheral chorion

laeve. The chorion frondosum can be mechanically separated from the surface of the decidua basalis at nine and one-half to ten and one-half days of gestation. This separation is not always as clean as might be desired and some adhering decidual cells frequently contaminated these cultures.

The physiological function of the fetal placenta is in part the production of choriogonadotropin for the maintenance of pregnancy. Huggett (1951, 1954) has shown that it is also the site of fructose synthesis from glucose in the embryos of ungulates, a role which is not completely understood. Although it may also be the site of fructose production in some rodents, the fructose concentration in the embryos of rodents is much smaller than in the ungulates. This investigation demonstrates that it is not essential in the mouse for the chorion frondosum to convert fructose into glucose before utilization by the extraembryonic tissues since each of the tissues apparently is able to carry out this conversion. The phosphohexoisomerase associated with this transformation in ungulate placentae has not been investigated thus far in mice. Fructose is normally converted into glucose in the liver of animals and a study of the mechanisms associated with an active reverse synthesis of this process may prove very interesting.

It is interesting that neither the chorion frondosum nor the allantois metabolized radioactive fructose very rapidly, especially since these are the structures associated with fructose synthesis in ungulate placentae. It would be

interesting to study more thoroughly the comparative rates of fructose and glucose utilization and synthesis in the fetal placentae of a number of mammals in different phylogenetic families.

Amnion.-The amnion arises as a double sheet of cells from the extraembryonic ectoderm and somatic mesoderm of the chorio-amniotic stalk at the periphery of the embryonic area. The amnion is nonvascular and might be expected to respire anaerobically in vitro; however, the amnion separating the exocoelom and amniotic cavity is in a position similar to that of the allantois for receiving oxygen for aerobic respiration. Since the autoradiograms and counting showed that progressively more glutamate and aspartate were synthesized with age of the amniotic cell at the time of explantation, the capacity for aerobic respiration evidently increased at the later stages. An explanation, perhaps, is that the disintegration of the decidua capsularis, parietal wall of the yolk sac, and Reichert's membrane during the later stages of gestation permits the oxygen to diffuse more readily from the uterine lumen into the embryonic exocoelom.

The amniotic fluid is not strictly a fetal product and it probably arises from the amnion cells since amniotic fluid is produced in many cases even if the embryo does not develop (Marshall, 1952). It has been suggested that the amnion protects the developing embryo from shock and desiccation, but its effectiveness in these roles is limited since in turn the amnion must also be protected from these physi-

cal factors (Marshall, 1952). The rapid turnover of water and salts in the amniotic fluid has been studied by Flexner and Gellhorn (1942); this process is undoubtedly controlled to a great extent by the semipermeability of the amnion. It was previously thought that the amniotic fluid was a stagnant pool; however, studies on the turnover rate of materials in the amniotic fluid have demonstrated the importance of the amnion for stabilizing the physiological contents of the amniotic fluid by discharging products of its metabolism into the amniotic fluid and by removing waste products of fetal metabolism from the amniotic fluids. Thus, the amnion provides a well stabilized aqueous medium in which the embryo may bathe and it may also synthesize directly nutrients for the developing embryo. It was formerly thought that the metabolic activity of the amnion was of little embryonic significance, but this no longer appears true.

The results of glucose and fructose metabolism for the synthesis of amino acids by the amnion are again quite different. It is obvious from Table 4 that the relative quantity of alanine synthesized from fructose metabolism is greatly diminished, whereas the relative amounts of serine and glycine are much larger. The reasons for these differences are not understood, but an effort will be made to explain them later.

Visceral yolk sac.-The yolk sac wall surrounding the yolk sac cavity arises as a double layer of cells from the extraembryonic splanchnic mesoderm and endoderm. It begins

to develop about the fifth day and is completely formed about the seventh day of gestation. The parietal wall of the yolk sac subsequently degenerates without much further development. The visceral wall becomes very large and the endodermal cells become metabolically very active during the growth and differentiation of the embryo. Its size and phylogenetic relationship with the yolk sacs of primitive mammals suggest that it functions as a placenta during early pregnancy. It accumulates large quantities of glycogen and lipids which are presumably absorbed from the decidua and are subsequently utilized to nurture the developing embryo. The nature of the absorption by the endodermal cells of the visceral yolk sac, studied with the use of dyes (Wislocki et al., 1946) and agglutinins (Brambell, 1954), suggests that it is capable of placental activities. The cytological details of the apical ends of the endodermal cells have been studied with the electron microscope and the presence of villiform processes and cryptiform invaginations communicating with a canalicular system indicates that pinocytosis occurs in these cells and that they are able to absorb nutrients for the embryo (Dempsey, 1953).

Histological observations show that the visceral yolk sac is the only hematopoietic organ functioning during early embryology in the mouse and that hematopoiesis within the liver and spleen of the embryo arises later. The number of macrophages in the cellular outgrowth obtained in cultures of the visceral yolk sac at various stages of develop-

ment is possibly correlated with its hematopoietic activity. The greatest number of macrophages, possible blood forming cells, was observed in cultures of the visceral yolk sac ranging from the eleventh day of gestation until the day before parturition. This is also the period of gestation during which hematopoiesis occurs in the visceral yolk sac (Table 2).

Although the yolk sac is vestigial in most animals with a highly developed allantoic placenta, the mouse is an exception. Moreover, this study on the growth and metabolism of pieces of yolk sac in vitro shows that the visceral yolk sac remains functional throughout gestation. It can be seen, however, that the synthetic activities of the visceral yolk sac change during later fetal life. The reduction of aspartate and glutamate synthesis as compared to serine and glycine in cultures of older yolk sac explants possibly results from the reduced vitelline circulation and consequent reduction in available oxygen. Wislocki et al. (1946) suggest that the visceral yolk sac metabolizes in an anaerobic atmosphere, but in vitro studies show that the cells in the yolk sac have the capacity for aerobic metabolism from the ninth day of gestation until parturition. There is a shift towards anaerobic respiration, as indicated by glucose utilization; however, this shift is not evident from fructose metabolism.

In the visceral yolk sac one also finds that during fructose metabolism much larger quantities of serine and

glycine are produced, while the metabolic activities giving rise to alanine are unfavorably affected. These results have been closely rechecked with repeated cultures of both the visceral yolk sac and the placenta and are highly reproducible. It was initially thought that the difference might in part be a result of differences in the periods of in vitro metabolism, the quantity of explanted tissue or age of the tissue, but these possibilities have been experimentally ruled out. There is a small possibility that some contaminating heavy metal or other enzymatic inhibitor was present in either the sample of U-glucose-C-1^4 or U-fructose-C-1^4 when it was purchased, but the nature of such a contaminant is not apparent. It is difficult to conceive of an inhibitor which could be so effective in such minute concentrations, as would be necessary if this were the case. Figure 1 shows that the U-fructose-C-1^4 was as nearly pure and free of radioactive contaminants as can be obtained commercially. The U-glucose-C-1^4 was less pure and contained some radioactive contaminants; the possibility that these contaminants are interfering has not yet been checked.

Biochemical aspects of embryonic metabolism.-When cellular metabolism is studied in vitro, the results must be interpreted with caution since the medium may change the normal activities of the cells. It is generally accepted that cells in tissue culture do not gain new capacities and it has been fairly well established from studies on pure cell strains that cells retain many of their capacities present

at the time of explantation for extended periods of time. For example, the LLCM1 mouse fibroblasts, after seventeen years of continuous in vitro culture, still retain their capacity to produce collagen fibers when placed in the proper culture conditions (Merchant, 1957). It may be assumed, therefore, that the biochemical activities measured in vitro are similar to those exhibited by the tissues in vivo. To be sure, in vivo conditions relating to oxygen tension, oxidation-reduction potentials, pH, substrates, and other regulatory factors are not always reproducible in vitro; in spite of this, studies of cellular activities under specified conditions have frequently been very helpful in elucidating normal metabolic activities. The biochemical pathways of metabolism have already been determined for many metabolites and this information may be applied to the interpretation of the autoradiograms obtained in this investigation. The lactate produced by these tissues apparently arose from the anaerobic phosphorylation and breakdown of the six carbon chain of glucose or fructose into three carbon units according to the generally accepted Embden-Meyerhof scheme of glycolysis. The quantity of glucose or fructose which may have been metabolized through the hexose monophosphate shunt in these tissues is unknown. Although the metabolism of fructose has not been investigated as thoroughly as glucose metabolism, present evidence is that almost all the fructose is metabolized by typical Embden-Meyerhof glycolysis; therefore, it is somewhat surprising to find that the patterns of metabo-

lism from glucose and fructose utilization in these tissues are quite different (Mann, 1949; Hers, 1955; Nakada, 1956).

Hers and Kusaka (1953) identified another pathway of fructose metabolism. Although fructose is ordinarily converted to fructose-1,6-diphosphate and metabolized by typical Embden-Meyerhof glycolysis, fructose-1-phosphate may also be formed and broken down directly into glyceraldehyde and dihydroxyacetone phosphate by a special aldolase. Although dihydroxyacetone phosphate is common to both Embden-Meyerhof glycolysis and the previously mentioned scheme of fructose metabolism, a unique compound, the nonphosphorylated glyceraldehyde, is produced only by fructose-1-phosphate metabolism. Since the initial formation of nonphosphorylated glyceraldehyde is the only recognized difference between glucose and fructose metabolism, it seems that nonphosphorylated glyceraldehyde may be a direct precursor for serine synthesis.

Glyceraldehyde can be phosphorylated by ATP and triokinase. The phosphoglyceraldehyde formed is a known precursor for serine synthesis, as will be discussed more extensively later. If this conversion generally occurs, equal amounts of phosphoglyceraldehyde would be produced from glucose and fructose; therefore, the concentration of phosphoglyceraldehyde cannot account for the difference found between glucose and fructose metabolism. It seems more probable that a direct two-step oxidation of the nonphosphorylated glyceraldehyde is occurring in these tissues yielding

hydroxypyruvate, which is subsequently aminated to produce serine. Additional experiments are being conducted to test this hypothesis.

The overall reactions for the production of energy arising from anaerobic glycolysis of glucose and fructose in the tissues may be expressed as follows: Each glucose or fructose unit plus two ATP, two phosphate, and two ADP yields two units of lactate plus four ATP. Therefore, two high energy phosphate units are gained from each unit of glucose or fructose metabolized anaerobically. It should be recalled that the energy gained from the anaerobic metabolism of each glucose unit derived from glycogen results in the production of three units of high energy phosphate, since the phosphorylation of glycogen does not require hexokinase and ATP but glycogen is phosphorylated by phosphorylase and inorganic phosphate. Thus the stored glycogen in the decidua or any other tissues may result in a very significant increase in energy when it is metabolized by the fetal tissues.

In 1954 Villee showed that slices of fetal tissues produced large quantities of lactate when incubated in a glucose-pyruvate medium. Harris (1956) reported that rat connective tissue cells in vitro exhibit a Pasteur effect (reduced glycolysis in atmospheres containing elevated partial pressures of oxygen), but still produce large amounts of lactate in aerobic environments. He also showed that cell division of rat fibroblasts is not inhibited under complete anaerobiosis in vitro. It might be thought that one of the

reasons for the failure for the blastocysts to multiply in vitro is their inability to metabolize anaerobically sufficiently to produce the necessary energy for cell multiplication. Indeed, this may have some importance but it is unlikely that it is the principal reason.

Alanine, serine, and glycine were the only amino acids synthesized in appreciable quantities during anaerobic metabolism. Alanine was probably produced by transamination of pyruvate, glutamate being the most common source of the labile amino group (Cohen, 1939, 1940). Glutamate plus pyruvate in the presence of pyridoxal phosphate and glutamic-pyruvic transaminase are in equilibrium with alpha-ketoglutarate plus alanine.

Serine probably serves as a precursor for the synthesis of glycine. Although serine may arise from glycine (Winnick, 1948), recent evidence shows that most of the serine derived from a carbohydrate source arises from 3-phosphoglyceric acid via 3-phosphohydroxypyruvate and phosphoserine (Adelberg and Rabinovitz, 1956). It was not possible to determine whether the serine was the source of glycine in these tissues since these two amino acids are interconvertible and an equilibrium is set up between them very rapidly. Nevertheless, since relatively more serine than glycine was formed from the radioisotopically labeled substrates by most of the tissues, it is at least possible that most of the glycine synthesized by the tissues arose from serine. One pathway was proposed by Shemin (1946) where

serine is reduced to a formylglycine intermediate and is subsequently dehydrated to yield formate and glycine.

The citric acid cycle is the aerobic portion of carbohydrate metabolism. Besides being an efficient means for synthesizing high energy phosphates, it is also a pool of precursors for carbohydrate, protein, and lipid metabolism. Several dicarboxylic organic acids are involved and two alpha-keto acids, oxalacetate and alpha-ketoglutarate, are the direct source of two amino acids, aspartate and glutamate respectively, by transamination. However, before concluding that these alpha-keto acids are the source of aspartate and glutamate, radioactive carbon derived from pyruvate metabolism should be found in these alpha-keto acids or in other acids of the citric acid cycle. In general, the autoradiograms showed that pyruvate was radioactive but, perhaps because of the relatively small quantity of the citric acid cycle constituents in tissues, the radioactivity present in these acids was frequently too minute to detect during short periods of autoradiography (Frohman et al., 1951). Autoradiography for extended periods of time revealed that radioactively labeled carbon was present in succinate (Figure 10) and this is evidence that the citric acid cycle is functional in these tissues and that it is a source of aspartate and glutamate.

Aspartate was most probably synthesized by transamination of oxalacetate, the source of the labile amino group probably being glutamate (Cohen, 1939, 1940). Gluta-

mate plus oxalacetate in the presence of glutamic-oxalacetic transaminase and pyridoxal phosphate are in equilibrium with alpha-ketoglutarate plus aspartate.

From the above reaction it can be seen that glutamate can be synthesized from alpha-ketoglutarate at the expense of the amino group of aspartate. Since the equilibrium of this reaction favors aspartate synthesis, it is not likely that aspartate is an important source of the amino group for the synthesis of glutamate. Since glutamate and aspartate are the principal sources of amino groups for the synthesis of other amino acids, it is important to note that glutamate and, to a lesser extent, aspartate take up nitrogen from ammonia or other amino acids, such as glycine or leucine, more readily than do other amino acids (Fruton and Simmonds, 1953). Thus, direct amination of alpha-ketoglutarate may supply many of the labile amino groups used for transamination.

The normal pathway for the synthesis of proline from glutamate is undoubtedly functioning since proline is synthesized only in those tissues which also synthesized large quantities of glutamate. Stetten (1951) and Vogel and Bonner (1954) showed how the straight carbon chain of glutamate can give rise to a cyclic compound such as proline by first being transformed into glutamic gamma-semialdehyde and pyrroline-5-carboxylate. Although the details of this conversion are not completely understood, it appears that a "proline oxidase" is necessary for converting the pyrroline-5-carboxy-

late to proline.

The pathway for the biosynthesis of histidine is not completely known. Ames et al. (1953) suggested that histidine was synthesized from pentose intermediates, but Levy and Coon (1954) found with the use of isotopically labeled substrates that perhaps pentose is not the precursor of the histidine synthesized by yeast. Glutamate may also be a precursor for histidine synthesis, but the pathway has not been completely elucidated.

It is important to recall that, since the citric acid cycle is a cyclic phenomenon, a small quantity of these intermediates can keep the cycle functioning. Nevertheless, transamination of the intermediates for the synthesis of amino acids would create an excessive drain upon the system if these intermediates were not replaced. Ochoa and coworkers (1948, 1950) found that carbon dioxide fixation to pyruvate, giving rise to malate, occurs in the presence of TPN; more recently, Utter and Kurahashi (1954) have shown that oxalacetate can be synthesized from phosphoenolpyruvate and carbon dioxide. These reactions operate independently and either reaction can effectively compensate for the alpha-keto acids removed from the citric acid cycle by transamination.

SUMMARY

1. The objectives of this investigation were to study various aspects of the metabolism of early mouse embryos, extraembryonic membranes, and uterine endometrium and to postulate from these studies possible interdependent and interrelated metabolic activities occurring among these tissues that may have significance in embryonic development.

2. The nature of the cellular outgrowth arising from tissue explants of mouse blastocysts, extraembryonic membranes, and uterine endometrium was studied in tissue culture.

3. Various media and culture vessels were used in an attempt to promote growth of the fastidious blastocysts. From the results obtained, hypotheses were made about the nature of the environmental factors and morphogenetic substances which are essential for blastocyst growth.

4. Uniformly labeled glucose (U-glucose-C-14) and U-fructose-C-14 were added to the culture media of four day old blastocysts, extraembryonic membranes explanted at various stages of development, and uterine endometrium explanted at estrus and also at four and one-half days after copulation. The metabolites formed from U-glucose-C-14 and U-fructose-C-14 were separated into two fractions---an alcoholic extract and an alcoholic precipitate. After appropriate pre-treatment

the metabolites in each fraction were separated and identified by two dimensional paper chromatography.

5. The radioactive metabolites were assayed both by autoradiography and with the aid of a geiger counter. The identified metabolites containing radioactive carbon derived from radioisotopically labeled glucose and fructose were lactate, pyruvate, succinate, alanine, serine, glycine, glutamate, aspartate, proline, histidine, cysteine, and an unknown ninhydrin-positive substance. These results were discussed in the light of probable biosynthetic pathways.

6. The data indicated that mouse blastocysts in vitro metabolized anaerobically, whereas all the other tissues demonstrated both aerobic and anaerobic metabolism in vitro.

7. The patterns of metabolism in vitro were used as a basis for postulating possible interdependent and interrelated metabolic activities occurring among these tissues in vivo. The yolk sac, which is vestigial in most mammals, was metabolically very active in the mouse even during the later periods of gestation and probably contributes significant amounts of nutrients for the fetus throughout gestation. It was formerly believed that the yolk sac became physiologically vestigial following the development of the chorioallantoic circulation.

8. The relative quantities of amino acids derived from U-glucose-C-14 and U-fructose-C-14 metabolism were very different.

9. The data demonstrated that the quantities of serine and, usually, glycine synthesized from U-fructose-C-14 were relatively greater than those from U-glucose-C-14, suggesting the existence of an additional previously unrecognized pathway for serine synthesis from a carbohydrate source.

Figure 1.-Autoradiogram illustrating the purity of U-fructose-C-14.

Figure 2.-Lactate synthesized by four day blastocysts from radioactively labeled glucose. In this culture fifty-nine mouse blastocysts were incubated for twenty-two days in a hanging drop plasma clot containing twenty microcuries of uniformly labeled glucose-C-14.

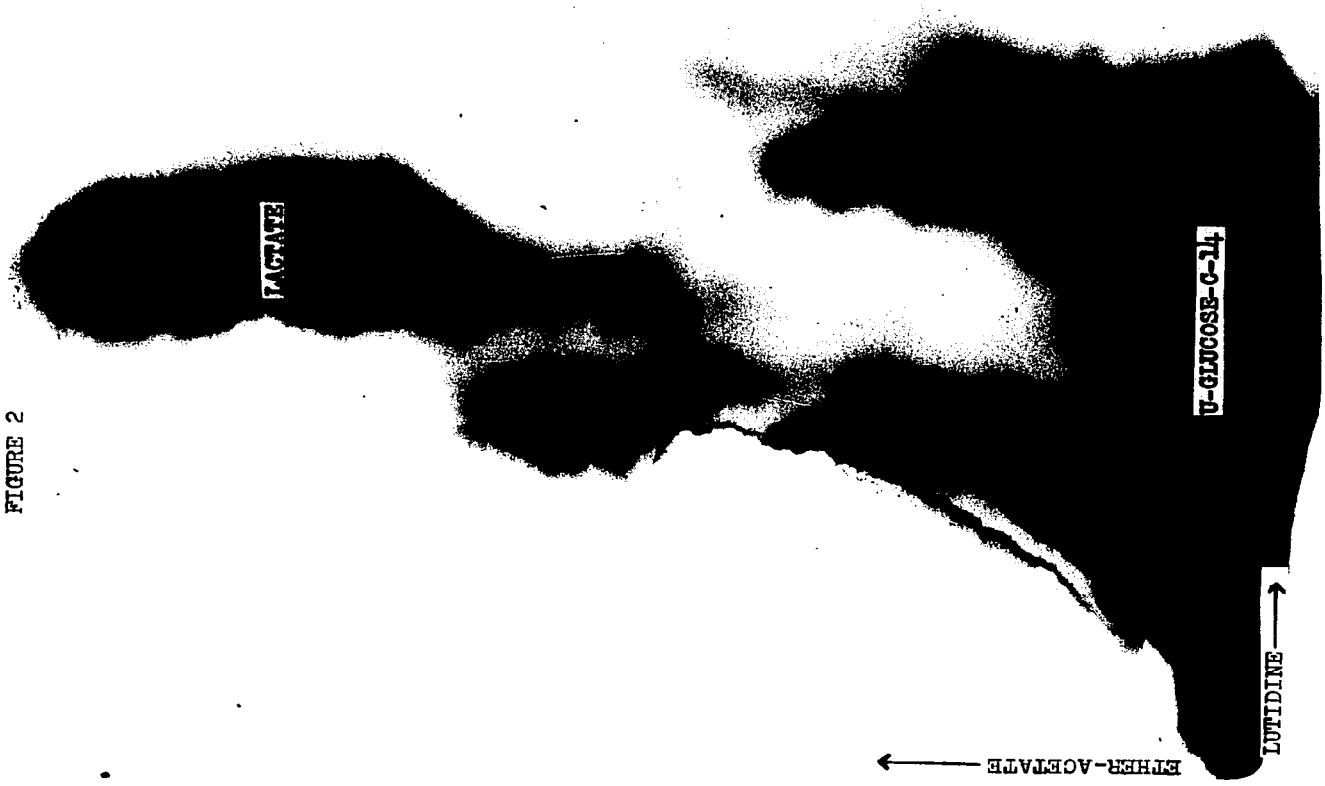


FIGURE 2



FIGURE 1

Figure 3.-Labeled amino acids derived from radioactively labeled glucose by the uterine endometrium at estrus. Roller tube culture incubated for twelve days in one milliliter of Parker's 703 medium containing ten microcuries of uniformly labeled glucose-C-14.

Figure 4.-Labeled amino acids derived from radioactively labeled fructose by nineteen day visceral yolk sac. Roller tube culture incubated for eight days in one milliliter of Parker's 703 medium containing ten microcuries of uniformly labeled fructose-C-14.

FIGURE 3

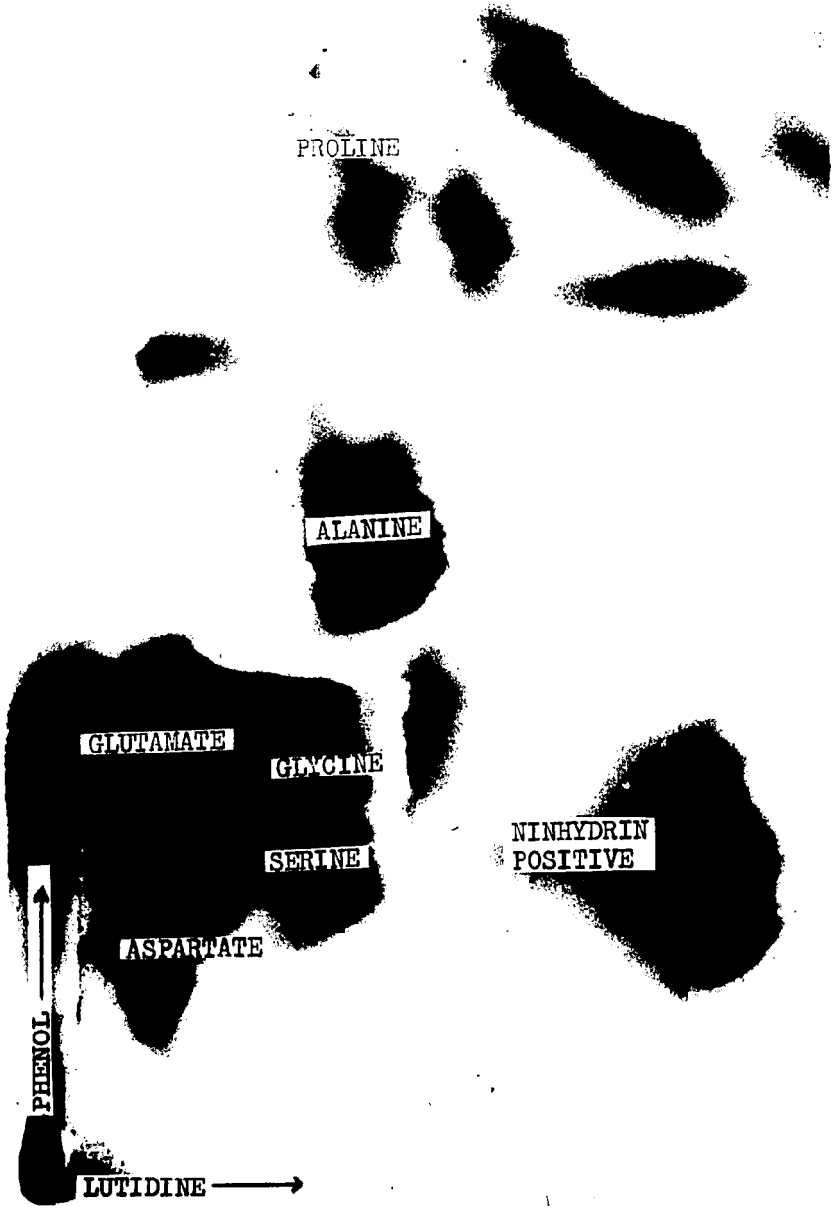


FIGURE 4

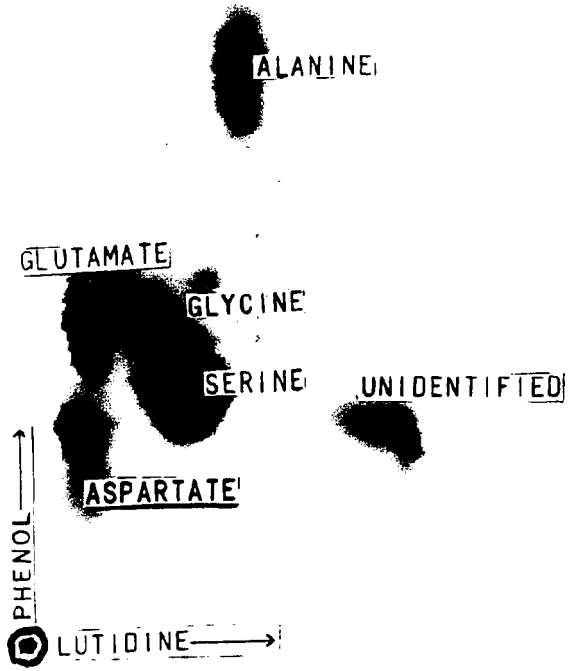


Figure 5.-Labeled amino acids derived from radioactively labeled glucose by the decidua capsularis explanted nine and one-half days after copulation. Roller tube culture incubated for ten days in one milliliter of Parker's 703 medium containing ten microcuries of uniformly labeled glucose-C-14.

Figure 6.-Labeled amino acids derived from radioactively labeled fructose by the decidua basalis explanted nine and one-half days after copulation. Roller tube culture incubated for six days in one milliliter of Parker's 703 medium containing ten microcuries of uniformly labeled fructose-C-14.

FIGURE 5

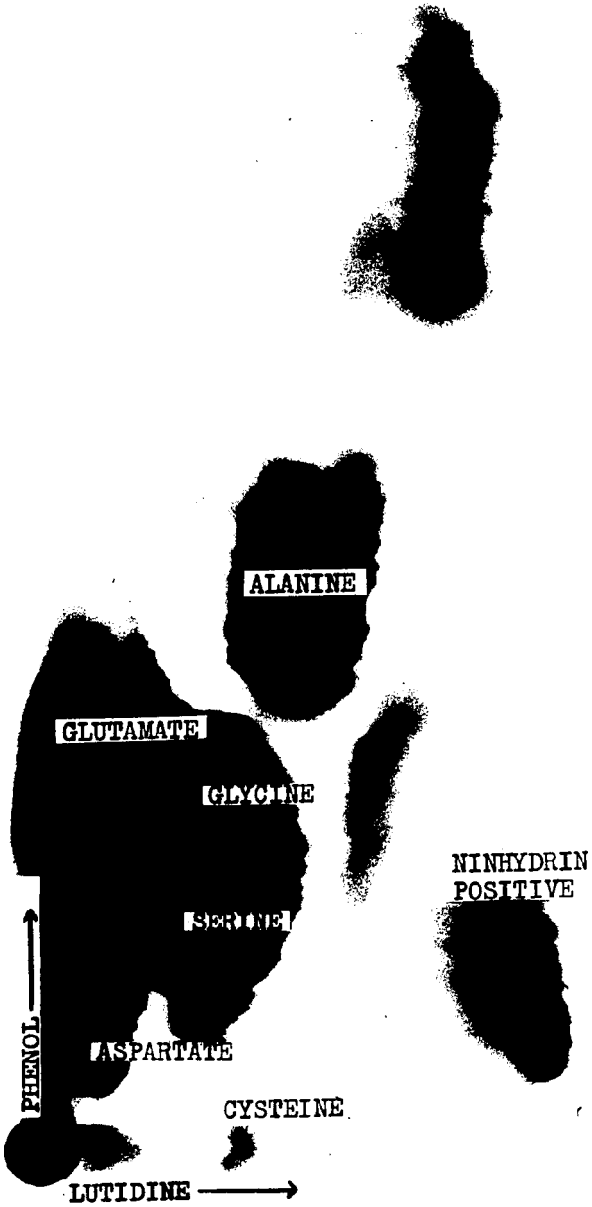


FIGURE 6

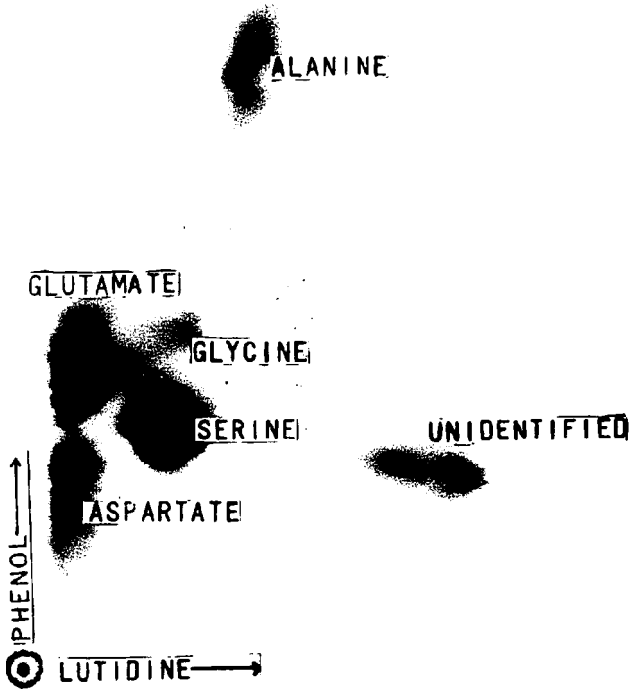


Figure 7.-Labeled amino acids derived from radioactively labeled glucose by thirteen and one-half day placenta. Roller tube culture incubated for ten days in one milliliter of Parker's 703 medium containing ten microcuries of uniformly labeled glucose-C-14.

Figure 8.-Labeled amino acids derived from radioactively labeled fructose by fourteen and one-half day placenta. Roller tube culture incubated for seven days in one milliliter of Parker's 703 medium containing ten microcuries of uniformly labeled fructose-C-14.

FIGURE 7

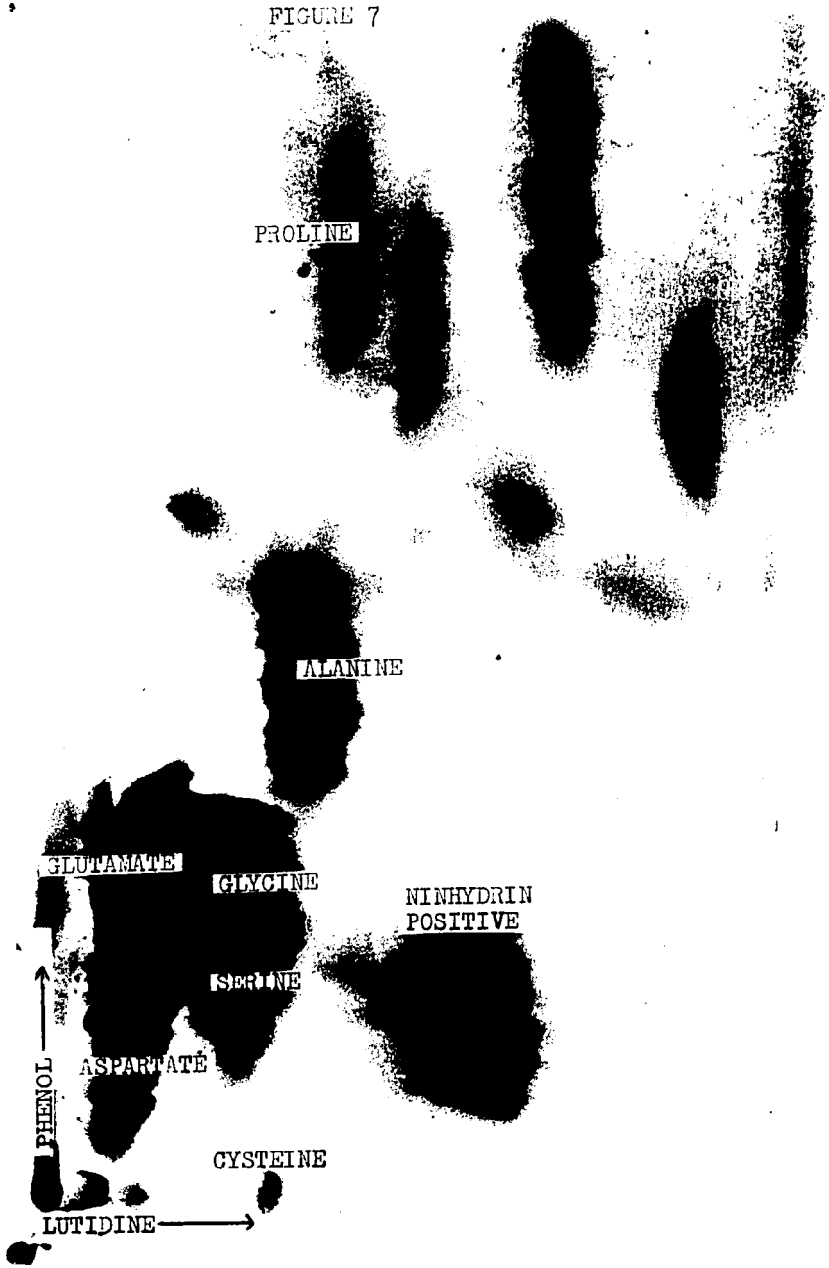


FIGURE 8

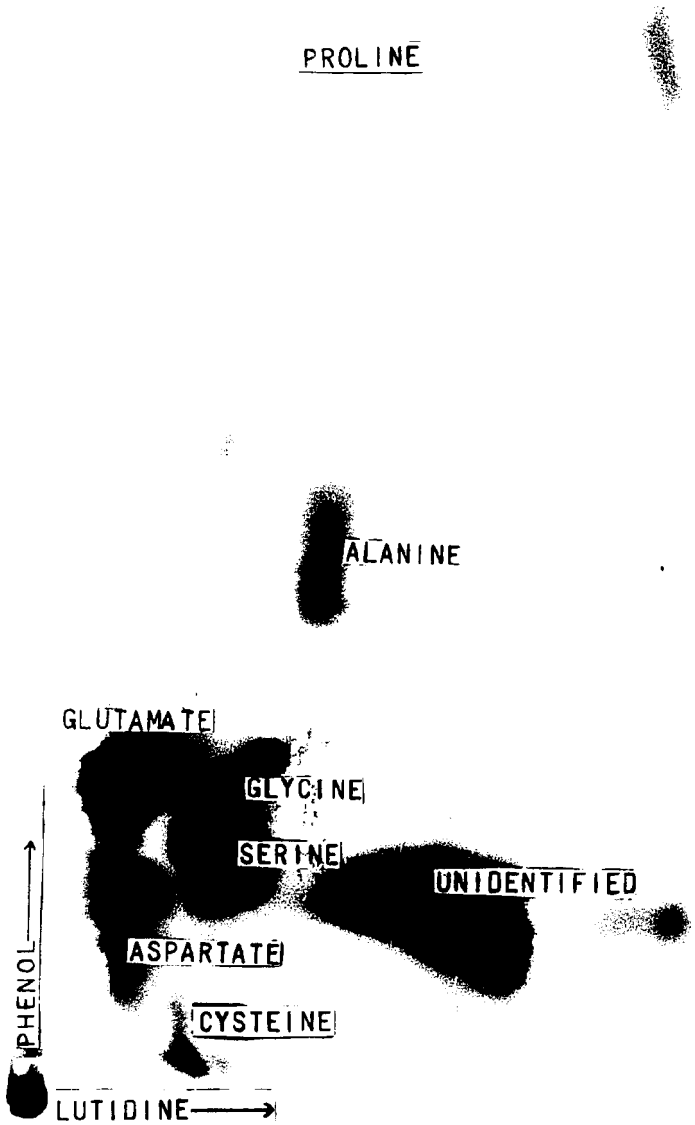


Figure 9.-Lactate synthesized by fourteen and one-half day amnion from radioactively labeled fructose. Roller tube culture incubated for thirteen days in one milliliter of Parker's 703 medium containing ten microcuries of uniformly labeled fructose-C-14.

Figure 10.-Lactate and succinate synthesized by nine and one-half day visceral yolk sac from radioactively labeled glucose. Roller tube culture incubated for twenty-one days in one milliliter of Parker's 703 medium containing ten microcuries of uniformly labeled glucose-C-14.

FIGURE 9



FIGURE 10

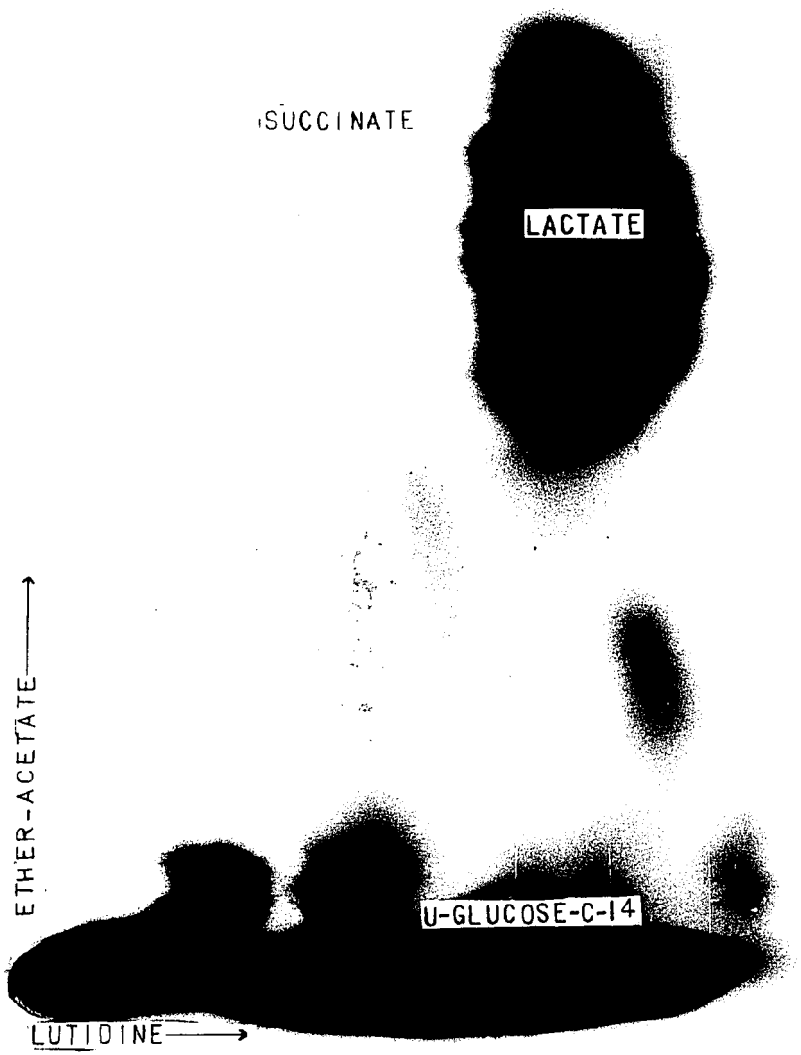


Figure 11.-Autoradiogram illustrating the amino acids formed by nine and one-half day visceral yolk sac from radioactively labeled glucose. In this culture nine and one-half day visceral yolk sac was incubated for four days in a hanging drop plasma clot containing five microcuries of uniformly labeled glucose-C-14.

Figure 12.-Chromatogram illustrating the corresponding positions of the labeled amino acids, as well as the unlabeled amino acids, identified with ninhydrin. This is the chromatogram from which the autoradiogram in Figure 11 was made.

FIGURE 11



FIGURE 12

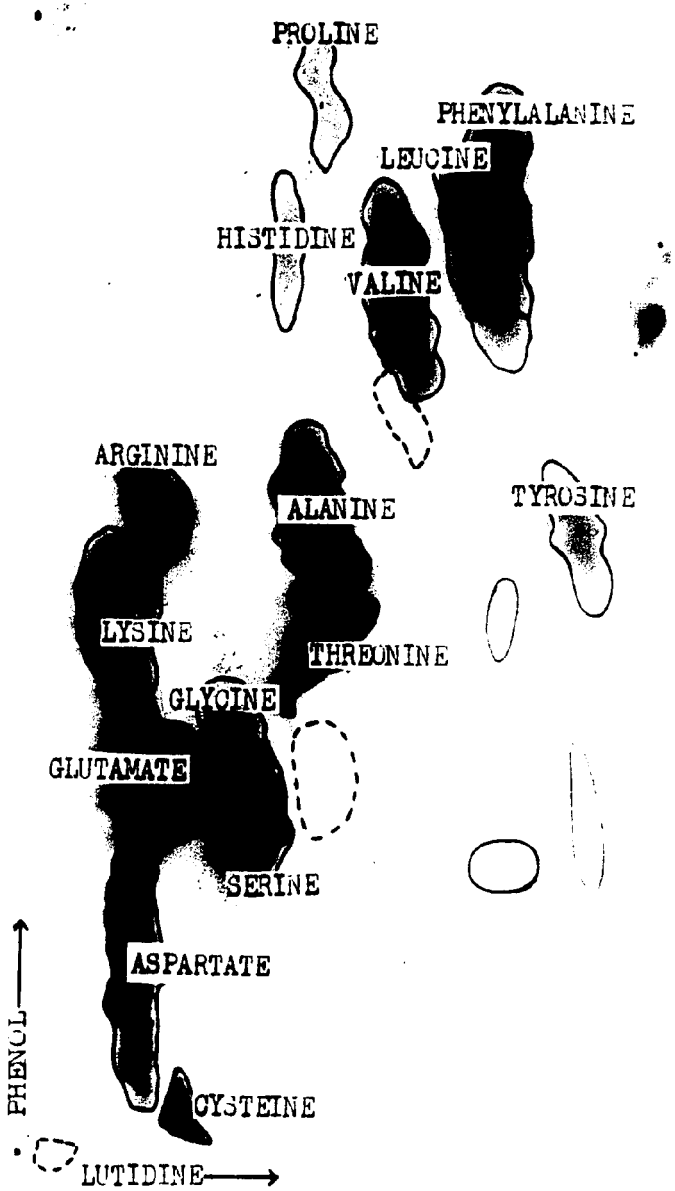


Figure 13.-Labeled amino acids derived from radioactively labeled glucose by thirteen and one-half day visceral yolk sac. Roller tube culture incubated for ten days in one milliliter of Parker's 703 medium containing ten microcuries of uniformly labeled glucose-C-14.

Figure 14.-Labeled amino acids derived from radioactively labeled fructose by fourteen and one-half day visceral yolk sac. Roller tube culture incubated for seven days in one milliliter of Parker's 703 medium containing ten microcuries of uniformly labeled fructose-C-14.

FIGURE 13

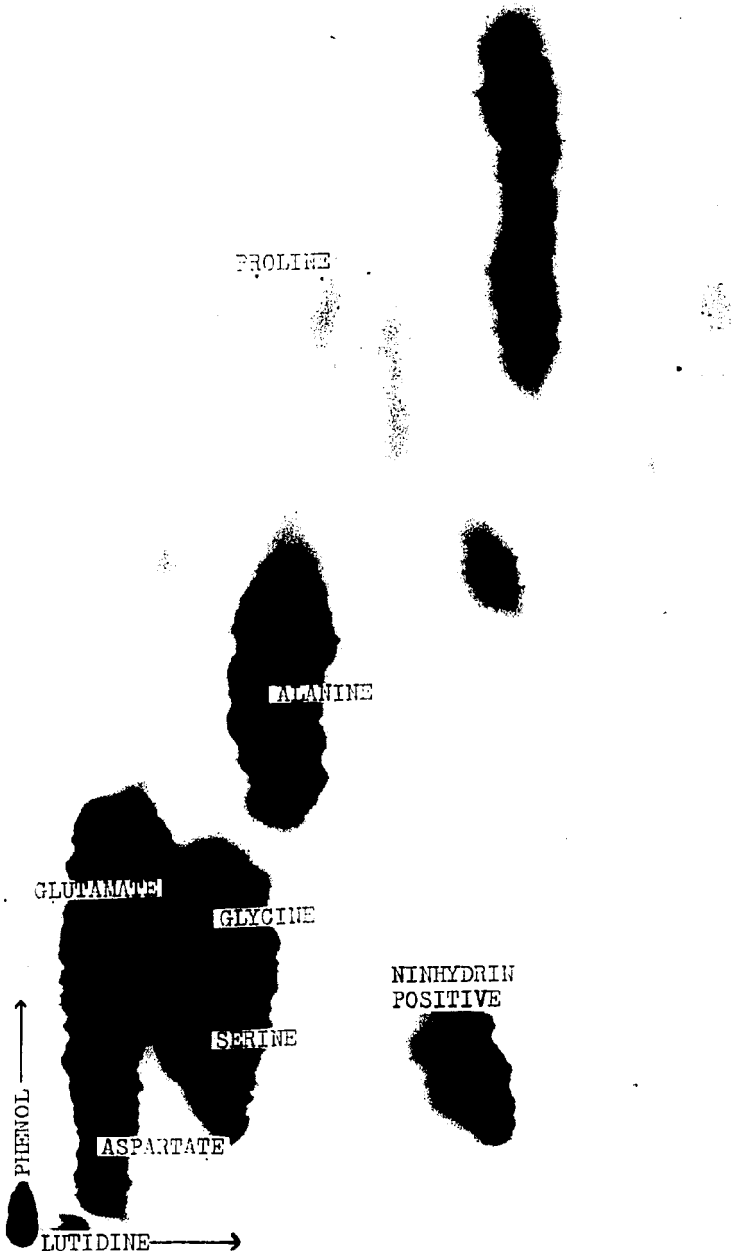
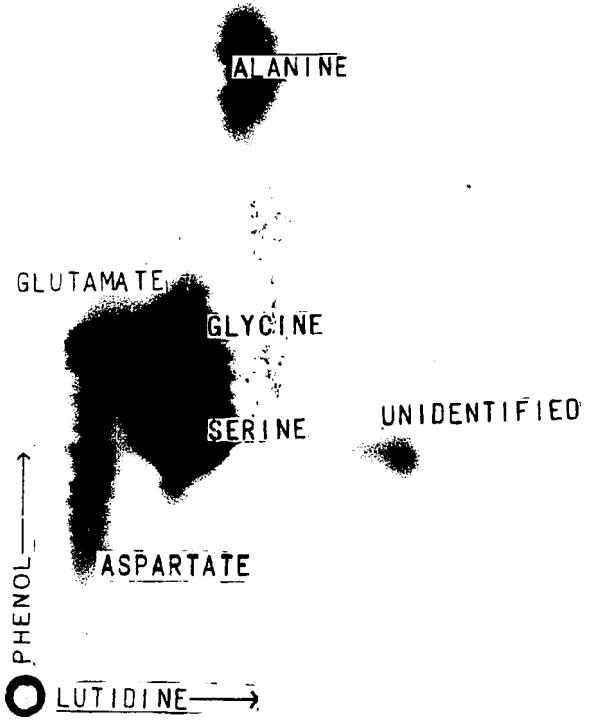


FIGURE 14



LITERATURE CITED

Adelberg, E., and M. Rabinovitz 1956 Metabolism of amino acids and proteins. Ann. Rev. Biochem. 25:349-372.

Alden, R. H. 1947 Implantation of the rat egg. II. Alteration in osmiophilic epithelial lipids of the rat uterus under normal and experimental conditions. Anat. Rec. 97:1-28.

Allen, W. M., and G. W. Corner 1929 Physiology of the corpus luteum. III. Normal growth and implantation of embryos after very early ablation of the ovaries, under the influence of extracts of the corpus luteum. Am. J. Physiol. 88:340-346.

Ames, B. N., H. K. Mitchell, and M. B. Mitchell 1953 Some new naturally occurring imidazoles related to the biosynthesis of histidine. J. Am. Chem. Soc. 75:1015-1018.

Awapara, J., and W. Wingo 1953 On the mechanism of taurine formation from cysteine in the rat. J. Biol. Chem. 203:189-194.

Baldwin, E. 1952 Dynamic Aspects of Biochemistry. Cambridge University Press, Great Britian.

Barron, E. S. G., and T. P. Singer 1945 Studies on biological oxidations. XIX. Sulfhydryl enzymes in carbohydrate metabolism. J. Biol. Chem. 157:221-240.

Beatty, R. A. 1951 Transplantation of mouse eggs. Nature 168:995.

Bell, W. B., L. Cunningham, M. Jowett, H. Millett, and J. Brooks 1928 The metabolism and acidity of the foetal tissues and fluids. Brit. Med. J. 1:126-131.

Berry, Helen K., and Louise Cain 1951 Quantitative study of urinary and salivary amino acids using paper chromatography. Biochem. Inst. Studies IV. Univ. of Texas Pub. 5109:71-76.

Berry, H. K., H. E. Sutton, L. Cain, and J. S. Berry 1951 Development of paper chromatography for use in the study of metabolic patterns. Biochem. Inst. Studies IV. Univ. of Texas Pub. 5109:22-55.

Bittner, J. J., and C. C. Little 1937 Transmission of breast and lung cancer in mice. J. Hered. 28:117-121.

Bloch, Suzanne 1939 Contributions to research on the female sex-hormones; implantation of the mouse egg. J. Endocrin. 1:399-408.

Borsook, H., H. W. Davenport, C. E. P. Jeffreys, and R. C. Warner 1937 The oxidation of ascorbic acid and its reduction in vitro and in vivo. J. Biol. Chem. 117:237-279.

Böving, B. G. 1954 Blastocyst-uterine relationships. Cold Spring Harbor Symposia on Quant. Biol. 19:9-28.

Brambell, F. W. Rogers 1954 Transport of proteins across the fetal membranes. Cold Spring Harbor Symposia on Quant. Biol. 19:71-81.

Briones, H., and R. A. Beatty 1954 Interspecific transfers of rodent eggs. J. Exp. Zool. 125:99-118.

Buch, M. L., R. Montgomery, and W. L. Porter 1952 On paper chromatography of organic acids. Anal. Chem. 24:489-491.

Burdick, H. O., and G. Pincus 1935 The effect of oestrin injections upon the developing ova of mice and rabbits. Am. J. Physiol. 111:201-208.

Carrel, A. 1912 On the permanent life of tissues outside the organism. J. Exp. Med. 15:516-528.

Chang, M. C. 1947 Normal development of fertilized rabbit ova stored at low temperature for several days. Nature 159:602-603.

_____ 1949 Effects of heterologous sera on fertilization of rabbit ova. J. Gen. Physiol. 32:291-300.

_____ 1950 Transplantation of rabbit blastocysts at late stage: probability of normal development and viability at low temperature. Science 111:544-545.

_____ 1951 Fertility and sterility as revealed in the study of fertilization and development of rabbit eggs. Fert. and Steril. 2:205-222.

Christensen, H. N., and J. A. Streicher 1948 Association between rapid growth and elevated cell concentrations of amino acids. I. In fetal tissues. J. Biol. Chem. 175:95-100.

Cohen, P. P. 1939 Transamination in pigeon breast muscle. Biochem. J. 33:1478-1487.

_____ 1940 Transamination with purified enzyme preparations (transaminase). J. Biol. Chem. 136:565-584.

Creighton, C. 1878 The formation of the placenta in the guinea pig. J. Anat. and Physiol. 12:534-590.

Dempsey, E. W. 1953 Electron microscopy of the visceral yolk-sac epithelium of the guinea pig. Am. J. Anat. 93:331-363.

Denison, F. W. Jr., and E. F. Phares 1952 Rapid method for paper chromatography of organic acids. Anal. Chem. 24:1628-1629.

Enders, R. K., and A. K. Pearson 1946 Mink blastocysts in vitro. Anat. Rec. 96:570.

Fawcett, D. W., G. B. Wislocki, and C. M. Waldo 1947 The development of the mouse ova in the anterior chamber of the eye and in the abdominal cavity. Am. J. Anat. 81:413-444.

Flexner, L. B., and A. Gellhorn 1942 The transfer of water and sodium to the amniotic fluid of the guinea pig. Am. J. Physiol. 136:757-761.

Fisher, A., G. Fischer, C. Lanshütz, G. Ehrenswärd, M. Rafelson Jr., and R. Stjernholm 1953 Amino acid formation from C^{14}-labeled glucose in cultures of fibroblasts from embryonic chicken heart tissue. Acta Physiol. Scand. 27:247-254.

Frohman, C. E., J. M. Orten, and A. H. Smith 1951 Levels of acids of the citric acid cycle in tissues of normal and diabetic rats. J. Biol. Chem. 193:803-807.

Fruton, J., and Sofia Simmonds 1954 General Biochemistry. John Wiley and Sons Inc., New York City.

Gerarde, H. W., M. Jones, and T. Winnick 1952 Protein synthesis and amino acid turn over in tissue culture. J. Biol. Chem. 196:51-68.

Gluecksohn-Schoenheimer, S. 1941 Development of early mouse embryos in the extraembryonic coelom of the chick. Science 93:502-503.

_____ 1944 The development of normal and homozygous brachy (T/T) mouse embryos in the extraembryonic coelom of the chick. Proc. Nat. Acad. Sci. 30:134-140.

Gregory, P. W., and H. Goss 1933 Glutathione concentration and hereditary body size. J. Exp. Zool. 66:155-171.

Grobstein, C. 1950 Production of intra-ocular hemorrhage by mouse trophoblast. J. Exp. Zool. 114:359-376.

Grüneberg, Hans 1952 The Genetics of the Mouse. Martinus Nijhoff, The Hague, Netherlands.

Hall, B. V. 1935 The reactions of rat and mouse eggs to hydrogen ions. Proc. Soc. Exp. Biol. Med. 32:747-748.

Hamond, J. Jr. 1949 Recovery and culture of tubal mouse ova. Nature 163:28.

Harris, H. 1956 The relationship between the respiration and multiplication of rat connective tissue cells in vitro. Brit. J. Exp. Path. 37:512-517.

Harrison, R. G. 1906-1908 Observations on the living developing nerve fiber. Anat. Rec. 1:116-118.

Healy, G. M., Dorothy C. Fisher, R. C. Parker 1954 Nutrition of animal cells in tissue culture. IX. Synthetic medium No. 703. Canad. J. Biochem. Physiol. 32:327-337.

Heape, W. 1890 Preliminary note on the transplantation and growth of mammalian ova within a uterine foster-mother. Proc. Roy. Soc. (Lond.) 48:457-458.

Hers, H. G. 1955 The conversion of fructose-1-C^{14} and sorbitol-1-C^{14} to liver and muscle glycogen in the rat. J. Biol. Chem. 214:373-381.

Hers, J. G., and T. Kusaka 1953 Le metabolisme du fructose-1-phosphate dans le foie. Biochim. et Biophys. Acta 11:427-437.

Huggett, A. St. G. 1954 The transport of lipids and carbohydrates across the placenta. Cold Spring Harbor Symposia on Quant. Biol. 19:82-92.

Huggett, A. St. G., F. L. Warren, and N. V. Warren 1951 The origin of the blood fructose of the foetal sheep. J. Physiol. 113:258-275.

Kirkham, W. B. 1916 The prolonged gestation period in suckling mice. Anat. Rec. 11:31-40.

Levy, L., and M. J. Coon 1954 Biosynthesis of histidine from radioactive acetate and glucose. J. Biol. Chem. 208:691-700.

Lewis, W. H., and E. S. Wright 1935 On the early development of the mouse egg. Contr. Embryol. Carneg. Instn. 25:113-144.

Lutwak-Mann, C., and H. Lasher 1954 Bicarbonate content of the blastocyst fluid and carbonic anhydrase in the pregnant rabbit uterus. Nature 173:268-270.

Mann, T. 1949 Metabolism of semen. Adv. Enzymology 9:329-390.

Markert, C. L. 1955 Substrate utilization in cell differentiation. Ann. N. Y. Acad. Sci. 60:1003-1014.

_____ 1956 The ontogeny of divergent metabolic patterns in cells of identical genotype. Cold Spring Harbor Symposia on Quant. Biol. 21:339-348.

Marshall, F. H. A. 1952 Physiology of Reproduction. Vol. II. Longmans, Green and Co., London, England.

Maximow, A. A. 1923 In vitro cultures of mammalian embryos. Anat. Rec. 25:141-142.

_____ 1925 Tissue culture of young mammalian embryos. Contr. Embryol. Carneg. Instn. 16:47-114.

Merchant, D. 1957 Personal communications.

Miller, B. J., and S. P. Reimann 1940 Effect of DL-methionine and L-cysteine on the cleavage rate of mammalian eggs. Arch. Path. 29:181-188.

Mossman, H. W. 1937 Comparative morphogenesis of the fetal membranes and accessory uterine structures. Contr. Embryol. Carneg. Instn. 26:129-246.

Nakada, H. I. 1956 The metabolism of fructose by isolated rat diaphragms. J. Biol. Chem. 219:319-326.

Needham, J. 1940 Biochemical Aspects of Organizer Phenomena. Growth Suppl. 4:45-52.

Nicholas, J. S. 1933 Development of transplanted rat eggs. Proc. Soc. Exp. Biol. Med. 30:1111-1113.

_____ 1934 Experiments on developing rats. I. Limits of fetal regeneration; behavior of embryonic materials in abnormal environments. Anat. Rec. 58:387-413.

_____ 1942 Experiments on developing rats. IV. The growth and differentiation of eggs and egg-cylinders when transplanted under the kidney capsule. J. Exp. Zool. 90:41-71.

Nicholas, J. S., and D. Rudnick 1933 The development of embryonic rat tissues upon chick chorioallantois. J. Exp. Zool. 66:193-256.

_____ 1938 Development of rat embryos of egg-cylinder to head-fold in plasma cultures. J. Exp. Zool. 78:205-232.

Ochoa, S., A. H. Mehler, and A. Kornberg 1948 Biosynthesis of dicarboxylic acids by carbon dioxide fixation. I. Isolation and properties of an enzyme from pigeon liver catalyzing the reversible oxidation decarboxylation of L-malic acid. J. Biol. Chem. 174:979-1000.

Padykula, H. A. 1952 The localization of succinic dehyrogenase in tissue sections of the rat. Am. J. Anat. 91:107-146.

Pincus, G., and J. Berkman 1937 Ascorbic acid during pregnancy in the rabbit. Am. J. Physiol. 119:455-462.

Pincus, G., and N. T. Werthessen 1938 The comparative behavior of mammalian eggs in vivo and in vitro. III. Factors controlling the growth of the rabbit blastocyst. J. Exp. Zool. 78:1-18.

Runner, M. N. 1947 Development of mouse eggs in the anterior chamber of the eye. Anat. Rec. 98:1-18.

Runner, M. N., and J. Palm 1952 Length of life of unfertilized mouse ova. Anat. Rec. 112:383.

_____ 1953 Transplantation and survival of unfertilized ova of the mouse in relation to postovulatory age. J. Exp. Zool. 124:303-315.

Sanford, K. K., R. E. Wilton, and G. D. Likely 1948 The growth in vitro of single isolated tissue cells. J. Nat. Can. Inst. 9:229-246.

Shemin, D. 1946 The biological conversion of L-serine to glycine. J. Biol. Chem. 162:297-307.

Simpson, S. A., and P. C. Williams 1948 Improved method of getting rats' eggs from the fallopian tubes. Nature 161:237.

Stetten, Marjorie R. 1951 Mechanism of the conversion of ornithine into proline and glutamic acid in vivo. J. Biol. Chem. 189:499-507.

Utter, M. F., and K. Kurahashi 1954 Mechanism of action of oxalacetic carboxylase. J. Biol. Chem. 207:821-841.

Veiga Sales, J. B., and S. Ochoa 1950 Biosynthesis of dicarboxylic acids by carbon dioxide fixation. II. Further study of the properties of the "malic" enzyme of pigeon liver. J. Biol. Chem. 187:849-861.

Villee, C. A. 1954 The intermediary metabolism of human fetal tissues. Cold Spring Harbor Symposia on Quant. Biol. 19:186-199.

Vogel, H. J., and D. M. Bonner 1954 On the glutamate-proline-ornithine interrelation in Neurospora crassa. Proc. Nat. Acad. Sci. 40:688-694.

Whitten, W. K. 1956 Culture of tubal mouse ova. Nature 177:96.

Winnick, T. 1952 Tissue culture methods in relation to biochemical studies. Texas Repts. Biol. Med. 10:452-462.

Wislocki, G. B., and E. W. Dempsey 1945 Histochemical reactions of the endometrium in pregnancy. Am. J. Anat. 77:365-403.

Wislocki, G. B., Helen W. Deane, and E. W. Dempsey 1946 The histochemistry of the rodent's placenta. Am. J. Anat. 78:281-345.

Wislocki, G. B., and Helen A. Padykula 1953 Reichert's membrane and the yolk sac of the rat investigated by histochemical means. Am. J. Anat. 92:117-151.