RNA AND PROTEIN SYNTHESIS DURING CYTODIFFERENTIATION IN FETAL RAT PANCREAS

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RNA AND PROTEIN SYNTHESIS DURING CYTODIFFERENTIATION IN FETAL RAT PANCREAS

By

John W. Bynum

AN ABSTRACT OF A THESIS

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ABSTRACT

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During cytodifferentiation in fetal rat pancreas epithelial cells with very little rough endoplasmic reticulum, a small Golgi apparatus and no zymogen granules differentiate into secretory cells with an extensive endoplasmic reticulum, an enlarged Grolgi apparatus and numerous zymogen granules. The accumulation of rough endoplasmic reticulum suggested that at some point during this period the fetal pancreas altered the rate at which it metabolized RNA, and this alteration resulted in the accumulation of ribosomes for the rough endoplasmic reticulum. This cellular transformation occurs between the 14th and 20th day of gestation. An increase rate of rRNA synthesis of a decreased rate of turnover could have caused such an accumulation. This study focuses on changes in the rate of RNA synthesis during cytodifferentiation.

Agarose-acrylamide slab gel electrophoresis was used to determine the amount of 28S, 18S, 5S and 4S RNA

at each embryonic age. The method involved homogenization of 100 to 400 µg of tissue in 100 µl of standard RNA extraction buffer (acetate, pH 5.1), 88% phenol and 0.5% SDS, extracting twice with phenol and final aqueous layer, directly on the gel. With this procedure, RNA degradation due to the high ribonuclease content of the pancreas was minimized and a quantity of RNA sufficient to give stained bands on gel was extracted from 19 µg of 15-day fetal pancreas. Electrophoretic profiles of RNA extracted from pancreatic rudiments of different age, showed no substantial difference in the amount or relative mobility of major RNA species throughout cytodifferentiation. RNA from older, more differentiated rudiments, with a higher content of ribonuclease was generally degraded more during extraction than RNA from younger rudiments.

Four modifications were made in developing a satisfactory organ culture system for fetal pancreases; the rudiments were cut into pieces of approximately 5-15 µg of protein to allow labelled precursors and nutrients to penetrate the interior of the tissue; the tissue pieces were attached to a Millipore filter as a substrate and flooded at the liquid gas interphase to facilitate oxygen-carbon dioxide exchange; fetal calf serum and supplemental amino acids were added to Eagle's MEM to provide additional precursors and growth factors; and antibiotics were added to inhibit bacterial contamination. RNA degradation was

used to evaluate tissue necrosis. Using the above modifications distorted RNA bands were resolved from RNA extraced from 17-day pancreas cultured for 24 hours. To provide a control for assessing differentiation and tissue survival, adult pancreas was cultured for 24 hours under the same conditions. RNA from adult pancreas indicated no tissue necrosis and the migration patterns were comparable to 17-day pancreatic RNA.

The rates of [^{3}H] leucine incorporation by pancreatic rudiments of different ages were determined at pH 6.8 and 7.1. At pH 6.8, the apparent rate of incorporation into TCA precipitable material (dpm/μg protein/hr) decreased between 14 and 15 days of gestation. The rate increased several fold to a maximum of 200 dpm/μg protein/hr at 17 days of gestation, then declined to the 15-day level after 20 days of gestation. The rate of protein synthesis in the adult pancreas was comparable to 18 and 19-day rates.

At pH 7.1, the apparent rate increased from approximately 150 dpm/μg protein hr at 15 and 16 days of gestation to a maximum of 450 dpm/μg protein hr, then declined to the 15-day level. The rates were approximately 3-fold greater than the pH 6.8 values at each age. Seventeen-day rudiments and adult pancreatic tissue were least affected by the change in pH.

When the apparent rates of protein synthesis were calculated per cell and plotted as a function of embryonic age, there was an apparent transition between 16 and 19 days of gestation at both pH 7.1 and 6.8. At pH 7.1 as well as 6.8, 19-day old tissue incorporated 8-fold more [^{3}H] leucine into protein per cell than did 15 or 16-day tissue. The greatest rate of protein synthesis per cell occurred in adult pancreases which were 2-fold greater than the 19-day rate at pH 7.1.

Similar incorporation studies were conducted with [^{3}H] uridine. Changing the pH from 6.8 to 7.1 caused only a slight increase in the apparent rates of RNA synthesis, although the apparent rates of protein synthesis were extensively affected. Again, in contrast to protein synthesis, the maximal rate in RNA synthesis occurred on day 18, one day before the transition in protein synthesis. From day 15 to day 18, the rate of RNA synthesis increased 5-fold at pH 6.8 and 2.5-fold at pH 7.1.

Calculating the rates of [^{3}H] uridine incorporation per cell instead of per microgram of protein did not alter the rate curve; maximal rates were attained after day 18 of gestation. Relative to DNA content, the apparent rate of RNA synthesis occurred a day before the maximal rate in protein synthesis; relative to protein content, the apparent maximal rate of synthesis occurred on the same day. These data, therefore, suggest that a transition

in both the apparent rate of RNA synthesis and the apparent rate of protein synthesis occurs in the embryonic rat pancreas midway through cytodifferentiation.

Electrophoredic analysis of [^{3}H] RNA from 14-day pancreatic rudiments indicated that within 4 hours of culture in labelled, peak corresponding to 28S and 18S RNA could be readily distinguished. After 8 hours of continuous labelling there was little variation in the distribution of radioactivity. After 24 hours the percentage of counts in 28S and 18S RNA increased 2-fold. The labelling ratio resembled the absorbance profile of total mammalian RNA. [^{3}H] RNA from 17-day pancreas gave radioactive profiles comparable to absorbance scans after 8 hours of culture. Electrophoresis of labelled 19-day pancreatic RNA gave results similar to 14-day pancreas. For all ages, the percentage of counts in 28S and 18S increased from 4 to 12 hours, while the percentage in 5S and 4S decreased from 4 to 24 hours. The 19-day pancreas differed from other ages in that at 4 hours 41% of the label was in low molecular weight RNA. As the labelling time continued, the percentage dropped to 21%. Seventeen-day pancreas also synthesized a high percentage of low molecular weight RNA early and then the percentage of counts in that region gradually declined.

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To my wife, Brenda, and daughter,
Sherrie, whose patience, sacrifice
and love made all this possible.

INVICTUS

Out of the night that covers me,
  Black as the Pit from pole to pole
I thank whatever gods may be
  For my unconquerable soul.

In the fell clutch of circumstance
  I have not winced nor cried aloud.
Under the bludgeonings of chance
  My head is bloody, but unbowed.

Beyond this place of wrath and tears
  Looms but the Horror of the shade,
And yet the menace of the years
  Finds, and shall find, me unafraid.

It matters not how strait the gate,
  How charged with punishments the scroll
I am the master of my fate;
  I am the captain of my soul.

--William Ernest Henley

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LIST OF ABBREVIATIONS

cpm: counts per minute

DNA: deoxyribonucleic acid

DPM: disintergrations per minute

EBSS: Earle's balanced salt solution

EDTA: ethylene diamine tetraacetate

GAT: poly-L-glutamine, L-alanine, L-tyrosine (60:30:10)

HnRNA: heterogenous nuclear ribonucleic acid

MEM: minimum essential medium

mRNA: messenger ribonucleic acid

PVS: polyvinyl sulfate

RNA: ribonucleic acid

rRNA: ribosomal ribonucleic acid

SAM: S-adenasyl methionine

SDS: sodium doderylsulfate

Stains-All: 1-ethyl-2-[3-(1-ethylnaptho-[1,2d] thiazolin-2-ylidene)-2-methylpropenyl]-naptho [1,2d] thiazolium bromide

TCA: tricholoracetic acid

TEAE cellulose: triethyamino ethyl cellulose

tRNA: transfer ribonucleic acid

MATERIALS

Materials were purchased from the following sources:

Agarose - Kinsman Optical Company

Ammonium persulfate - Fischer Scientific Company

Antibiotics - 10,000 units/ml of penicillin and 10,000 µg/ml of streptomycin - Grand Island Biological Company

L-arginine - Sigma Chemical Company

Bio-Gel P-30 - Bio Rad Laboratories

Bovine serum albumin (35% sterile) - Sigma Chemical Company

Bovine serum albumin - Sigma Chemical Company

Chick Embryo extract - Grand Island Biological Company

Chloramine T - Eastman Organic Chemicals

Cyanogum 41 - Fischer Scientific Company

3-dimethylaminopropronitrile - Eastman Ogranic Chemicals

Deoxyribonuclease - Worthington

Eagle's minimum essential medium - Grand Island Biological Company

Fetal calf serum - Grand Island Biological Company

Formamide - Aldrich Chemical Company

L-glutamic acid, L-alanine and L-tyrosine polymer (60:30:10) - Pilot Chemical Division of New England Nuclear

L-glutamine (200 mM) - Grand Island Biological Company

Heparin - Sigma Chemical Company

Iodine (^{125}I) - New England Nuclear

[^{3}H] leucine (25 Ci/mM) - Amersham/Searle Corporation

L-methionine - Nutrition Biochemicals Corporation

Pancreas ribonuclease (bovine) - Sigma Chemical Company

Peptone - Sigma Chemical Company

Platicware (tissue culture) - Falcon Plastic Company

Polyvinyl-sulfate - General Biochemicals

Pronase - Calbiochem

Proteose peptone - Difco Laboratories

Sprague Dawley rats - Spartan Research Animals

Stains-All - Eastman Organic Chemicals

TEAE cellulose - Sigma Chemical Company

[^{3}H] uridine (25 Ci/mM) - New England Nuclear

Yeast RNA - Sigma Chemical Company

PART I

INTRODUCTION

Current trends in biological research have turned increasingly toward the regulation of cellular differentiation in higher organisms. A portion of this interest is due to growing concern over the molecular basis of such publicized disorders as cancer and organ transplant rejections, and the need to confirm the existence of similar bacterial metabolic phenomena in mammalian systems. However, a substantial number of the myriad laboratories pursuing differentiation are involved in the delineation of the controls and functions of the antibody producing system consisting of the lymphocytic and monocytic series (macrophages).

Lymphocytic Series

The Lymphocytic series consists of a family of cells having common properties and sharing the function of defending the body against invasion of bacteria and other noxious agents. The induction of this system is initiated by a series of events beginning with the activation of precursor stem cells to differentiate; and terminates with the production of cell capable of removing the extraneous factor by direct cellular contact or the assembly and

secretion of antibody. As later references to current publications will show, investigation of this process has been prolific on both the cellular and molecular levels.

A large amount of evidence has accumulated suggesting that small lymphocytes are the precursors of antibody producing cells (1, 2, 3). Papermaster postulates that small lymphocytes originate in bone marrow from stem cells which are equally capable of becoming erythrocytes, granulocytes, or lymphocytes. The key factors in determining the developmental path are the cellular environment and the availability of certain hormones. Erythropoietin is indicated as the hormone responsible for committing the stem cell to a pathway of erythropoiesis (4). Failure of thymectomized mice to produce antibody after injections of bone marrow cells denotes the importance of the thymus in developmental lymphopoiesis (3).

Further evidence for the function of the thymus has developed around two physiological interactions; one is hormone production and the other is lymphocyte distribution. Repeated injections of thymus extract restored the immunological reactivity of thymectomized new-born mice (49.50), but animals so treated remained lymphopenic in certain regions of the lymph nodes and spleen (51). Consequently, the thymus may have a dual function in that it also seeds lymphocytes to the spleen and lymph nodes. In mice progeny of thymus lymphocytes can be identified

by the presence of a specific antigenic site called the "theta" antigen (53). These are referred to as "T" lymphocytes (54).

Studies of lymphocyte function in birds revealed another organ which could compliment or possibly duplicate the activities of the thymus. This organ is the bursa of Fabricus, which lies adjacent to the cloaca (55). Investigations for a site comparable to the bursa in mammals indicates the existence of such tissue, but as yet, its exact location is unknown. Possible sites are the Peyer's patches, bone marrow and the appendix. In rabbits removal of the gut-associated lymphoid organs followed by a lethal dose of radiation resulted in immunological characteristic similar to a bursectomized bird (56). Lymphocytes which do not seem to be thymus dependent but which may be under the influence of a "bursa" are currently called "B" lymphocytes (66).

After the lymphocytes are distributed to the peripheral lymphoid organs, further specialization appears to be independent of hormonal control. At this stage the lymphocyte has become sensitive to the physiological surroundings, and responds only to certain factors. These include mitogenic agents (phytohemagglutin (5, 6), antigens (3, 7, 8), antigen-antibody complexes (9), and macrophages (10, 11, 12, 13). Within minutes after stimulation with these agents, heterogenous and soluble RNA is

synthesized and ribosomal precursor RNA and ribosomal RNA gradually increase (14). The lymphocyte acquires a blast cell morphology resembling the precursor stem cell (15, 16). Through a mitotic transformation, the blast cell divides to form more small lymphocytes capable of further differentiation (17). The labelling patterns shown by small lymphocytes have recently indicated that two classes can result from the transformation of the blast cell. One class of lymphocytes is long-lived; the other is short-lived and proportionately more responsive to induction (18). During subsequent development, the cell is again confronted with what appears to be alternative division paths. One path involves a transformation into plasma cells consisting of enlargement and development of the structures necessary for rapid protein synthesis and secretion, rough endoplasmic reticulum and Golgi apparatus (19). After several divisions and the achievement of maturity, the plasma cell is phagocytized (20). An alternate but seemingly unlikely pathway (21) consists of a transformation into macrophages (22).

Macrophages

The term macrophage is used to define a family of cells which is capable of phagocytosis or engulfment of foreign particulate material. These phagocytes are the reticuloendothelial system. Macrophages have been found

in a variety of tissues in a wide physiological distribution: for example, the peritoneal macrophage of the peritoneal cavity (23), the aveolar macrophage of the lung (24), the splenic macrophage of the spleen (25), the Kupffer cell of the liver (31), the microglia cell of the central nervous system (32), the lymphoid macrophage of the thymus and lymph nodes (33), the monocyte of the blood (34). Due to the chemotaxis, macrophages are found in a number of other tissues. However, those listed above appear to be the major foci of macrophage populations.

Macrophages differentiate through a series of transitions from precursor cells found in the bone marrow (35, 36, 37). The first differentiated stage of exudate macrophages, which are found in lesions, involves the monocyte which is present in the circulating blood (38). During the course of inflammation the monocyte first leaves the vascular system and migrates into the extravascular connective tissue to the site of inflammation. Under normal conditions, this migration is random and usually terminates in the connective tissue. The factors regulating precursor differentiation and monocyte emigration are currently unknown. In the second stage of differentiation the monocyte in the connective tissue of the inflammatory site matures into a macrophage; the mature macrophages then remain within local sites and engage in phagocytic functions (39). Although the intermediate cell

types have not been delineated, macrophages in other locations also have been shown to be derived from cells in the bone marrow (40).

"Immunogenic" RNA

Macrophages aid significantly in immunity because they phagocytize and process antigen. This processed antigen is apparently coupled to ribonucleic acid (immunogenic RNA) and transferred to the precursors of antibody forming cells (41, 42, 43, 44, 45). A class of "immunogenic" RNA composed of the synthetic antigen poly L-glutamic acid, L-tyrosine and L-alanine (60:30:10) has been isolated and partially characterized (46). The ribonucleoprotein complex was found to band in cesium sulfate at a density of 1.588 grams per cc, to have an $S_{20,w}$ of 1.8 and an approximate molecular weight of 12,000. After removal of the polypeptide, the $S_{20,w}$ was 1.3 and the guanine cytosine content was 58% (47). After fractionation of the macrophages, the antigen RNA complex was distributed between the supernatant fraction and the ribosomal fraction in a ratio of 2:1. An antigen RNA complex from poly-γ-D glutamic acid was stable to sucrose gradient centrifugation, adsorption chromatography, gel electrophoresis and equilibrium sedimentation in cesium sulfate. Furthermore, the labelled polypeptide was not dissociated by 10^5 fold excesses of unlabelled polypeptide.

There was no association between the RNA and polypeptide mixed *in vitro*, but association occurred with a cell-free homogenate (57). A similar complex was obtained with polypeptide mixed with homogenate from HeLa and *E. coli* cells. It may be that RNA antigen binding is a nonspecific ionic interaction in that the complex was not found in RNA extracted by the hot phenol method (58). In competition studies, it was found that synthetic polypeptides made from L-amino acids were bound to RNA eight to ten times as well as D-amino acid polymers *in vitro*. This effect was attributed to the resistance of D-polypeptides to catabolism (68). Similar studies conducted with synthetic polypeptides of different electrical charges revealed that positively charged L-amino acid polymers were in higher concentration in the RNA fraction than were negatively charged polymers. Neutral polymer were associated to the least extent (69).

Immunogenic RNA has also been isolated from liver, spleen and lymph nodes. By assessing the distribution of labelled BSA injected into rabbits, it was found that the antigen BSA was mainly localized in the spleen, lymph nodes and the liver. Of the three tissues, liver retained the highest percentage of antigen for the longest period (59). Cellular studies showed that an antigen RNA complex in liver RNA could induce rapid antibody synthesis in normal spleen cells *in vitro*. Treatment of the complex

with ribonuclease abolished its immunogenicity, while degradation of the protein portion with pronase did not affect the immunogenicity (60). This suggested that immunogenicity resides in the RNA and not the protein and that the protein is possibly functioning as a carrier or nuclease inhibitor.

In the work of Dray, nonimmune rabbit spleen cells were converted by RNA of lymphoid cells from an immune rabbit to produce antibody of foreign heavy and light chain allotype (61, 62). The results indicated that a component of the RNA extract provided information for synthesis of at least part of the heavy chain as well as the light chain of the IgM and IgG antibody molecule. Johnson demonstrated a regulatory action of poly A:C on the immune response (63, 64). Polynucleotide binding stimulated the production of membrane bound IgG molecules in an anamnestic response (65).

Research Approach

Increasing evidence points to the existence of a unique species of RNA in macrophages and macrophage containing tissues which regulates cellular and humoral immunity. Gottlieb isolated such a component from peritoneal macrophages using TEAE cellulose chromatography and the synthetic antigen [^{125}I] GAT as a marker (46). I have attempted to utilize his procedures in isolating an

antigen-RNA complex from peritoneal exudate cells and other tissues of the reticuloendothelial system. In pursuit of this problem, I have investigated cell induction in the peritoneal cavity of rats and evaluated antigen uptake and retention by these cells. Using chromatography and polyacrylamide gel electophoresis, I have found no evidence to support the existence of a unique low molecular weight RNA species in these tissues.

METHODS

Isolation of Splenocytes

Dissociation of rat spleen gave a suspension rich in cells from the lymphocytic and monocytic series with minor contamination by the other component of peripheral blood. However, in the isolation of spleen cells, the nature of the disruption produced contaminants such as connective tissue and cell aggregates which prevented preparation of pure macrophages. To alleviate this problem, techniques were sought which would more thoroughly dissociate the cells and more effectively remove the stroma. All manipulations were performed under sterile conditions. The number of cells was approximated with a hemacytometer.

Splenocytes were isolated from tissue treated by three different procedures: mincing, mincing and filtration, and mincing and gentle homogenization.

Mincing

Sprague Dawley of various ages and rats were decapitated and the spleen aseptically transferred to a beaker containing 5 ml of sterile Earle's balance salt solution (EBSS). The cells were dissociated by mincing the tissue with scissors until a paste-like suspension

was formed. Large tissue aggregates were removed by transferring the suspension to a conical centrifuge tube and allowing them to sediment for five minutes. Smaller cell aggregates were removed by transferring the upper 7/8 (vol.) of the suspension to a conical centrifuge tube and allowing them to sediment for 10 minutes. Cells were collected by transferring the upper 7/8 (vol.) of the suspension to a conical centrifuge tube and pelleting in a clinical centrifuge.

Mincing and Filtration

The tissue was treated as in mincing but instead of sedimenting, the suspension was forced through a Swinney adaptor (Millipore Corp.) containing a 4000 mesh nylon screen to remove small cell aggregates.

Mincing and Homogenization

The tissue was treated as in mincing. To further dissociate the cell aggregates, the suspension was homogenized by ten strokes of a modified, large clearance, hand homogenizer. A cell suspension in that the 15 ml Ten Broeck tissue grinder completely disrupted the tissue to subcellular fragments. A modified homogenizing vessel with a clearance sufficient for cell dissociation was made from a 65 mm polypropylene powder funnel with a 25 mm stem bored to accommodate a 150 x 16 mm test tube.

The test tube was shortened by 15 mm to allow the pestle from the 15 ml Ten Broeck grinder to reach the bottom. The funnel provided a basin for the test tube.

Spleens were excised and dissociated by mincing, mincing and filtration, and mincing and gentle homogenization. Mincing yielded 2.2×10^7 cells per spleen with substantial contamination by cell aggregates and connective tissue (Table 1). Mincing and filtration yielded 2.2×10^7 cells per spleen but with very little contmination. Mincing and gentle homogenization yielded 4.2×10^7 cells per spleen with a large number of cell clumps and pieces of stroma. Consequently, a procedure involving mincing, followed by gentle homogenization and then filtration of the resulting cell suspension was found to be the most desirable cell isolation procedure.

Isolation of Glass Adhering Cells

Splenocytes were cultured for 24 hours in complete medium: 89% (v/v) Eagle's minimum essential medium (MEM) containing Hank's balanced salt solution, 10% (v/v) fetal calf serum, 100 units/ml of penicillin and 100 µg/ml of streptomycin. 1.7×10^7 cells were added to 100 x 20 mm plastic dishes containing 10 ml of medium and incubated in a moist atmosphere of 5% carbon dioxide and 95% air (standard incubation conditions). Unattached cells were removed after 1 hour incubation with a pipette and the

Table 1. Summary of methods of preparing splenocytes. Rat spleen was dissociated by different methods and purity and yield of splenocytes were assessed by microscopy.

Method	Cell Yield/Spleen	Contamination
Mincing	2.2×10^7	Cell clumps, connective tissue, subcellular debris
Mincing and filtration	2.2×10^7	Subcellular debris
Mincing and homogenization	4.2×10^7	Cell clumps, connective tissue subcellular debris

attached cells washed with EBSS and evaluated. Similar procedures were followed using cover slips inserted in Leighton tubes.

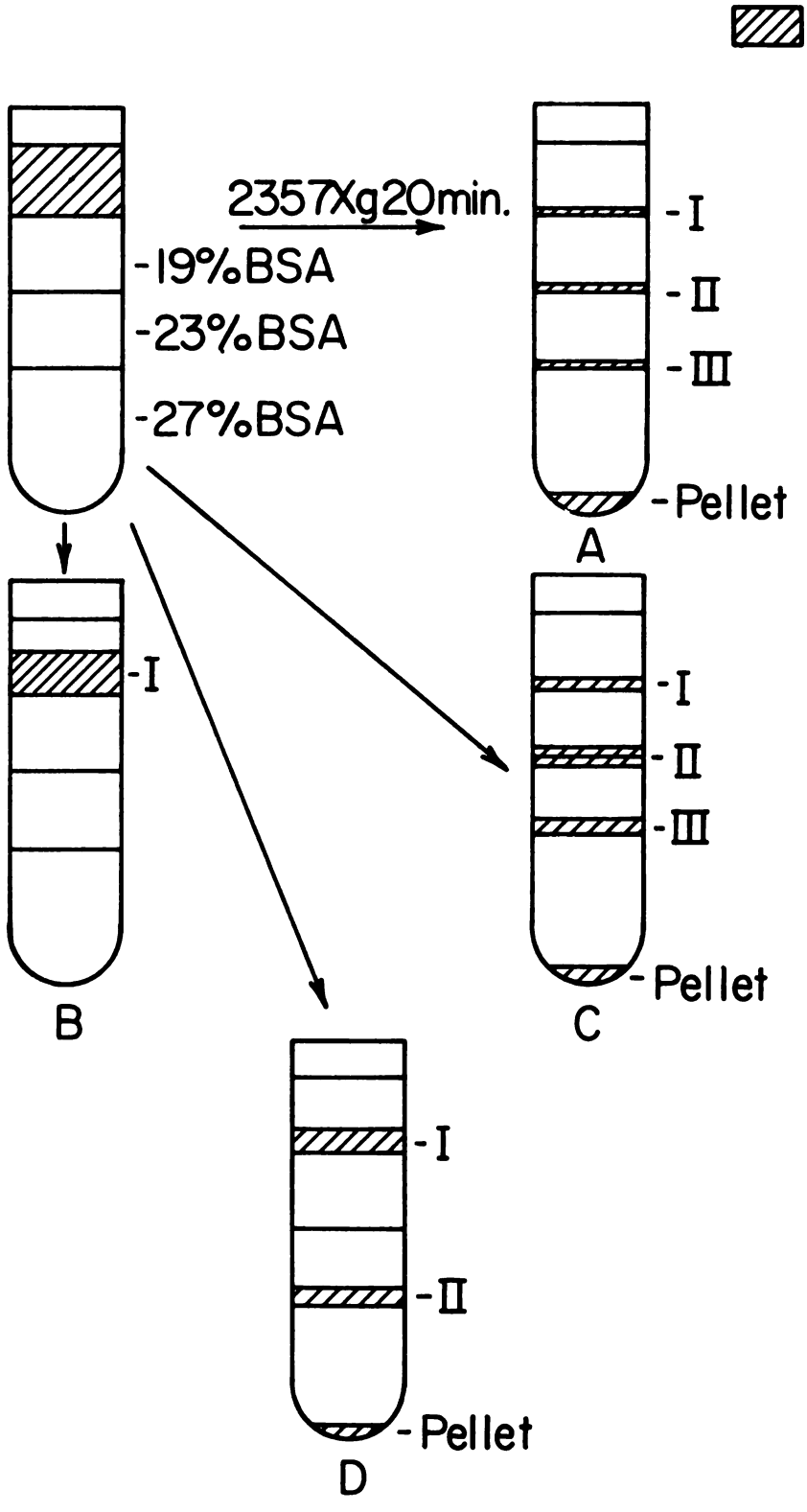
Splenocytes were cultured for 24 hours to allow macrophages and monocytes to attach to the plastic surface. The attached cells had the morphology of macrophage, but the yield of cells was low as compared to other methods.

Density Separation of Splenocytes

The density separation procedure used was a modification of the technique of Bennett and Cohn (26). A 35% sterile solution of bovine serum albumin (BSA) was diluted with EBSS to make a discontinuous gradient. Splenocytes at a concentration of 2×10^7 cells/ml in a volume of 1 ml were layered over a discontinuous gradient of 1 ml of 19% BSA, 1 ml of 23% BSA and 2 ml of 27% BSA. The gradient was centrifuged at 2,357 x g for 20 minutes in a Sorvall refrigerated centrifuge with a swinging bucket rotor (HS-4). Cells of macrophage morphology banded at the interphase of 23% and 27% BSA. Similar gradients were utilized with brain, liver and lung cell suspensions. The suspension of splenocytes layered over the discontinuous BSA gradient and sedimented was separated into three distinct bands and a pellet (Figure 1A). Band I, at the interphase of the layering

Figure 1. Density separation of cell populations by sedimentation. Suspensions of various tessues were layered over a discontinuous gradient of BSA and sedimented 2,357 x gravity for 20 minutes as described in METHODS. Spleen is A, brain is B, lung is C and liver is D. BSA concentrations used were 27%, 23% and 19%.

DENSITY SEPARATION OF CELLS



solution and 19% BSA and composed mainly of cellular debris and particles the size of mitochandria. Band II was located at the interphase of 19% and 23% BSA and contained clusters of cells and undissociated tissue. Band III, located at the interphase of 23% and 27% BSA, contained cells of macrophage morphology, red blood cells and lymphocytes. The pellet contained mostly lymphocytes and red blood cells with some macrophages. The cells in Band III were sedimented and counted. The yield was 5.97% of the sedimented cells: 90% were macrophages. After 8 days in culture, splenocytes gave only a pellet upon sedimenting. Macrophages and lymphocytes were the major cell types in the pellet.

To determine whether the liver, lung and brain contained cells of similar density, suspensions of these tissues were prepared and sedimented using the procedure for spleen. Brain tissue gave a single band at the interphase of the layering solution and 19% BSA (Figure 1B). Lung tissue sedimented into three bands and a pellet (Figure 1C). Band I contained membranes and organelles, Band II was composed of small lymphocytes, connective tissue and septal cells, and Band III was mostly debris with a few lymphocytes. In the pellet red blood cells were the predominant type. The liver suspension sedimented into two bands and a pellet (Figure 1D). Bands I and II were cellular debris, and the pellet contained tissue clumps

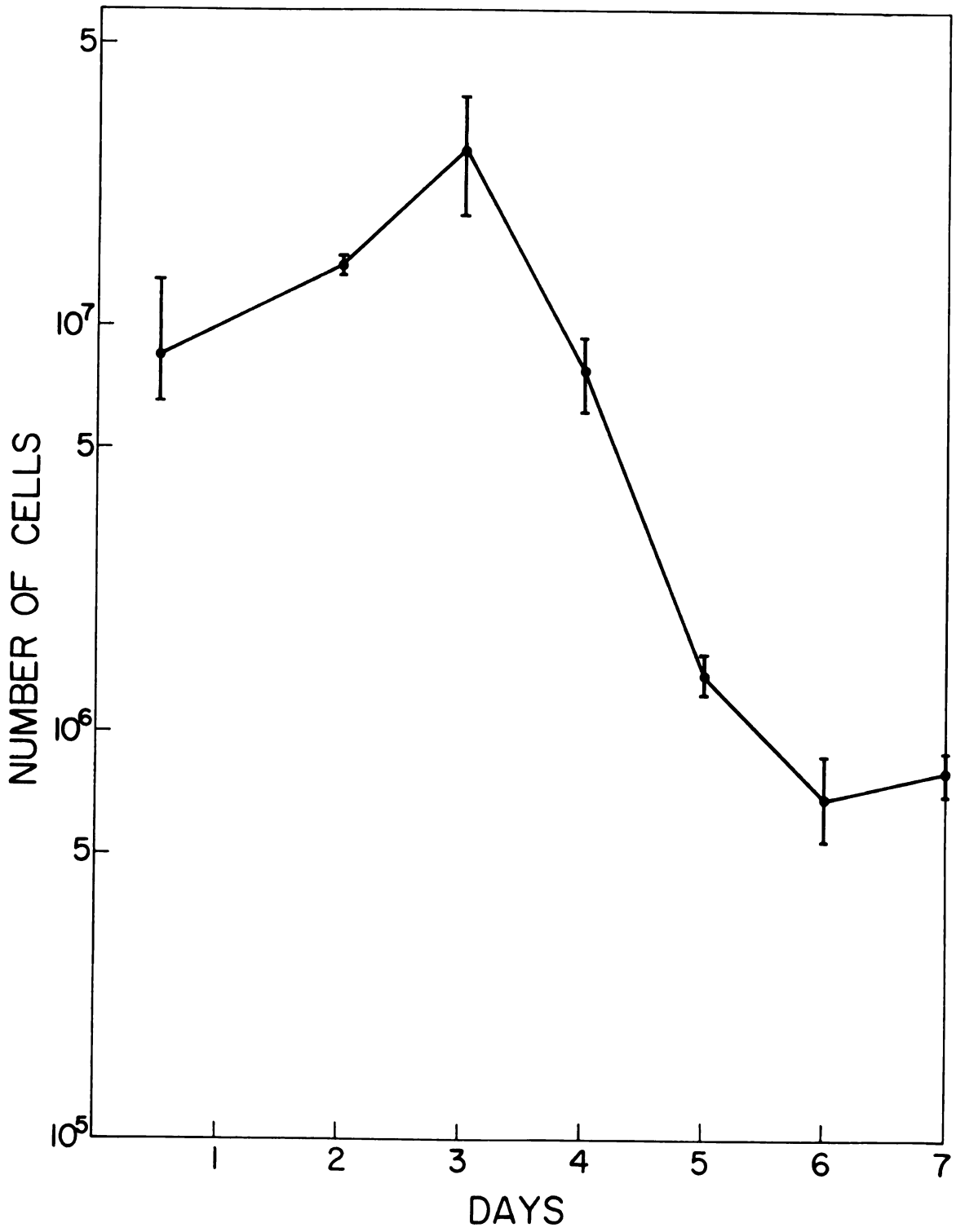
and red blood cells. This indicated that the monocyte from spleen is the only phagocyte which sediments at a BSA density comparable to blood monocytes (26).

Macrophage Induction in the Peritoneal Cavity

Several investigators reported that relatively pure populations of peritoneal macrophages could be induced in four days with pyrogenic solutions of protein or mineral oil. To confirm these reports, the following experiments were conducted to find a pyrogenic solution and a time after pyrogen injection which induced the greatest number of macrophages in the peritoneal cavity of rats.

In an attempt to determine the time necessary for the maximum induction of cells in the peritoneal cavity, rats were injected with 10 ml of 50% mineral oil in 10% peptone and the accumulation of cells in the cavity was evaluated. The number of cells in the cavity reached a maximum on the third day with a gradual decline and leveling off by the sixth day (Figure 2). Macrophages were more prominent on the fourth, fifth and sixth day as judged by the number of cells with the characteristic morphology. Consequently, the fourth day was selected as the appropriate time to harvest the optimum number of cells having the greatest percentage of macrophages.

Figure 2. Induction of peritoneal cells in rats. Rats were injected intraperitoneally with 10 ml of a solution of 50% mineral oil and the accumulation of cells in the peritoneal cavity was evaluated at various times after the injection.


NUMBER OF CELLS
5
10^7
5
10^6
5
10^5
1
2
3
4
5
6
7
DAYS

Rats were injected intraperitoneally with 10 ml of various pyrogenic solutions to determine which gave optimal cell induction: 10% peptone in EBSS, 50% mineral oil in EBSS, 50% mineral oil in 10% peptone and 50% mineral oil in proteose peptone. Four days after the injections the rate were sacrificed. Thirty milliliters of EBSS containing 5 units/ml of heparin were injected into the peritoneal cavity and the resulting exudate aspirated. The cells were counted and compared to control injections of EBSS. All mineral oil solutions were sonicated to make an emulsion. Recovery of exudate from the cavity was 67% to 83% of the 30 ml injected (Table 2). Peptone and 50% mineral oil gave the highest cell yield. Rats injected with proteose peptone and mineral oil were dead by the third day, half of the rats died on the second day after the injection. The symptoms displayed before death suggested anaphylaxis.

Iodination of GAT

A random synthetic polymer of L-glutamic acid, L-alanine and L-tyrosine (GAT) with a molecular weight of approximately 55,000 was radioiodinated according to a modified procedure of Greenwood, et al. (27). The reaction mixture contained 50 µl of carrier free $Na^{125}I$, 30 µg of GAT (1 mg/ml), 25 µl of 0.5 M sodium phosphate (pH 7.5) and 15 µl of the catalyst 7 mM choloramine T in 0.05 M

Table 2. Summary of cell induction by pyrogenic solutions. Rats were injected in the peritoneal cavity with 10 ml of various pyrogenic solutions. After four days, rats were sacrificed by anesthethization and decapitation, and the cells in the peritoneal cavity were counted.

Injection Solution	No. of Expts.	Total Cells Removed	Volume Removed	Cells/ml of Exudate	Total Cells in Cavity	Percent Recovery	Average No. of Cells in Cavity
10% Peptone	1	7.26×10^5	23 ml	3.15×10^4	9.45×10^5	76.6	
	2	1.23×10^6	20 ml	6.15×10^5	1.8×10^7	66.6	9.47×10^6
Mineral Oil	1	1.86×10^6	20 ml	1.43×10^5	4.2×10^6	66.6	
	2	3.86×10^6	25 ml	1.54×10^5	4.6×10^6	83.3	4.4×10^6
10% Peptone and 50% Mineral Oil	1	9.43×10^6	20 ml	4.7×10^5	1.4×10^7	66.6	
	2	6.09×10^6	24 ml	2.54×10^5	7.6×10^6	80.0	1.08×10^7
EBSS	1	4.2×10^5	21 ml	2×10^4	6×10^5	70	
	2	3.96×10^5	21 ml	1.88×10^4	5.6×10^5	70	5.8×10^5

sodium phosphate (pH 7.5). The reaction time was varied according to the concentration of GAT approximately three minutes per 10 µg of polymer. The reaction was stopped by adding 50 ml of 12.6 mM sodium metabisulfite in 0.05 M sodijm phosphate buffer, pH 7.1. A solution of 0.047 M sucrose, 0.023 M potassium iodide and 0.1 mg/ml of bromphenol blue was added to the reaction mixture and the resulting solution transferred to a 1 x 32 cm Bio Gel P-30 column equilibrated with 0.005 M sodium phosphate buffer, pH 7.1. The reaction vessel was washed with 100 µl of a rinsing solution similar to the transfer solution containing only 0.023 M sucrose. The polymer was eluted with 0.05 M sodium phosphate buffer in 50 drop fractions (approximately 1 ml). Aliquots were counted in a Packard scintillation counter and the absorbance at 230 nm in each fraction was measured to determine the elution profile of labelled polypeptide.

Several reaction times and polymer concentrations were employed in an effort to incorporate more ^{125}I into GAT. The time and concentration chosen had to be sufficient to allow maximum iodination of the tyrosine residues without deaminating the amino acids in the polymer. Reaction times of 8 and 10 minutes with 20 and 30 µg of polymer, respectively, were found to incorporate over 50% of the label into GAT.

The labelled polymer was separated from free $NA^{125}I$ on a Bio Gel P-30 column having an exclusion limit of 30,000. The radioactivity elution profile indicated that no labelled components of a molecular weight between those of the exclusion limit and the iodide salt (Figure 3). Based upon absorbance at 230 nm four peaks were eluted most of Peak I eluted with the radioactivity peak of GAT. Peak III contained the bromphenol blue marker in addition to the radioactive iodide salt. Peak II eluted near the GAT peak and may have been fragment of polymer lacking tyrosine. Peak I did not completely coincide with the [^{125}I] GAT which could have been due to a contaminant in the presence of polymer containing little tyrosine. The recovery of label added to the Bio Gel column was evaluated by adding a known amount of [^{125}I] GAT to reaction mixtures having varying quantities of unlabelled polymer. Using three separate columns, the recovery was consistently 71% (Table 4).

[^{125}I] GAT Uptake by Cells from the Peritoneal Cavity

Phagocytic cells were induced in the peritoneal cavities of rats by intraperitoneal injections of 5 to 10 ml of 50% mineral oil in 10% peptone sonicated to make an emulsion. Three to four days later the rats were sacrificed and the cells harvested. Rats were sacrificed by

Figure 3. Separation of radioiodinated poly GAT from Na ^{125}I by gel filtration chromatography. Labelled iodine (Na ^{125}I) was reacted with 30 µg of GAT for 8 minutes and the reaction mixture was fractionated on a 1 x 32 cm Bio Gel P-30 column equilibrated with 0.05 M sodium phosphate buffer (pH 7.5). The absorbance at 230 nm was determined in a Gilford spectrophotometer and 10 ml aliquots were evaluated for radioactivity by scintillation counting. Fractions were collected in 50 drop aliquots (approximately 1 ml). A maximum absorbance in Peak III, bromphenol blue, was reached at fraction 10.

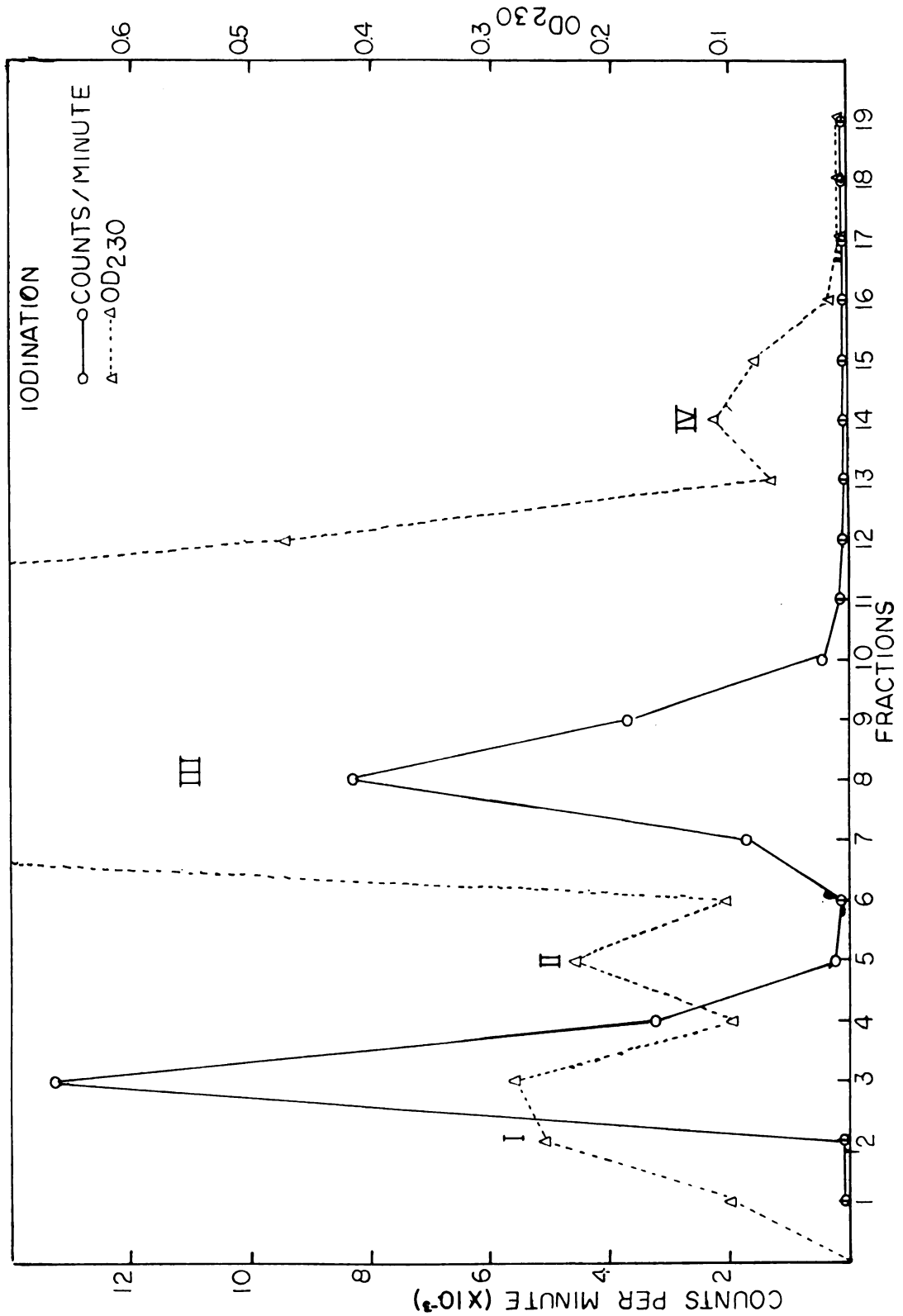

IODINATION
COUNTS/MINUTE
OD230
I
II
III
IV
COUNTS PER MINUTE (X10⁻³)
FRACTIONS
OD230

Table 3. Summary of [^{125}I] GAT recovery from Bio Gel P-30 columns. Iodine reaction mixtures containing 7.1 x 10^4 cpm of iodinated polymer were added to a 1 x 32 cm Bio Gel P-30 column and the recovery from the column was determined.*

µg of GAT in Reaction Mixture	Counts/min. Recovered	% Recovery
10	60,845	71
20	63,195	71
30	66.651	71

*70,976 cpm was added to each column.

a) cervical dislocation and decapitation, b) ether asphyxiation, and c) anesthethization and decapitation to determine which method gave the lowest contamination by red blood cells. Anesthetization and decapitation gave the least red blood cell contamination. The cells were washed several times in EBSS to remove residual mineral oil and resuspended in sterile MEM. The cell yield was approximately 5×10^5 white cells per rat with 80 to 95% of the cells having macrophage marphology.

Aliquots of cells were mixed with complete medium containing 0, 5 and 10% fetal calf serum and dispensed in 1 ml volumes in 12 x 75 mm sterile plastic centrifuge tubes or 10 x 30 mm plastic dishes. After various periods of incubation under standard conditions, the cells were sedimented, washed with MEM and cell number and the content radioactivity was determined. Similar procedures were followed with red blood cells isolated from peripheral blood.

[^{125}I] GAT Retention by Peritoneal Cells

The retention of ^{125}I by peritoneal cells *in vitro* was measured by adding 3 ml of cells in MEM (8.7×10^6 cells/ml, 20% white cells) to 2 ml of [^{125}I] GAT (2.5 µg/ml at 8.9×10^4 cpm/µg) in 2.5% BSA and 0.05 M phosphate buffered physiological saline (pH 7.5) and incubating

under standard conditions for 12 hours. The cells were harvested and washed and the radioactivity in the cells and incubation medium determined.

[^{125}I] GAT Stability

The extent of degradation of [^{125}I] GAT during cell culture as well as after storage at -20°C was evaluated by gel filtration on chromatography on a 1 x 32 cm Bio Gel P-30 column equilibrated with 0.05 M phosphate buffer 9pH 7.5). One ml samples were applied to the column and 50-drop fractions were collected at room temperature.

RNA Extraction

Rats were sacrificed and the liver, brain, lung, spleen and pancreas were excised and frozen on dry ice. The frozen tissue was placed in a chilled semimicro blender container with 25 ml of acetate EDTA buffer containing 0.01 M sodium ethylenediamine tetraacetate (EDTA), 0.01 M sodium acetate buffer (pH 5.1) and 5 μg/ml of polyvinyl sulfate (PVS), and 30 ml of redistilled phenol. Sodium dodecyl sulfate (SDS) was added to a final concentration of 1%. The tissue was homogenized for four 30-second intervals with interim 60-second periods of chilling in a 0°C ice bath. The phases were separated by centrifugation and the aqueous phase re-extracted twice with an equal volume of phenol. Residual phenol was removed by ether

extraction and the ether evaporated by bubbling the aqueous phase with nitrogen. The RNA was precipitated twice in 0.1 volume of 1 M NaCl and 2 volumes of ethanol at -20°C for 4 hours. All glassware was acid or base washed to reduce ribonuclease contamination.

TEAE Cellulose Chromatography of Low Molecular Weight RNA

Low molecular weight RNA was separated on a 1 x 24 cm TEAE-cellulose column equilibrated with 0.02 M Tris-Cl (pH 8.2) and eluted with a linear gradient of 0.1 to 0.75 M sodium chloride in Tris buffer. The column eluent was monitored with an ISCO ultraviolet analyzer and recorder. The concentration of sodium chloride was monitored with a conductivity meter (Radiometer Copenhagen) and the absorbance at 280 nm was determined with a Model 240 Gilford spectrophotometer.

Gel Electrophoresis

Gel electrophoresis was conducted in the vertical analytical cell of Raymond, E-C470 according to the method of Peacock (29, 29, 30).

Gel Buffer

A stock solution of Tris EDTA borate (10X) buffer (pH 8.3) containing 0.89 M Tris, 0.89 M boric acid and 0.025 M disodium EDTA was prepared and diluted as needed.

Slab Gels

The gels used were either agarose acrylamide composite gels or straight acrylamide gels. Agarose acrylamide composite gels were 2% acrylamide and N,N'-Methylenebisacrylamide (bis), and 0.5% agarose and were used to analyze total or high molecular weight RNA. The acrylamide and bis used were purchased as Cyanogum 41 in a ratio of 19:1. Gels were prepared from 160 ml of gel solution contained 0.8 g of agarose, 109 ml of water, 16 ml of 20% Cyanogum 41, 10 ml of 6.4% 3-dimethylaminopropronitrile, 16 ml of 10 x Tris EDTA borate buffer and 5 ml of 1.6% ammonium persulfate.

The acrylamide gels used were either 5% or 7.5% acrylamide-bis. The gel solution varied in that it contained 69 ml of water and 60 ml of Cyanogum 41 for 7.5% gels, and 89 ml of water and 40 ml of Cyanogum 41 for 5% gels. Acrylamide gels did not contain agarose.

Cylindrical Gels

Cylindrical gels were prepared according to Loening (48) with recrystallized bis and acrylamide. A gel solution of 7.5% acrylamide (w/v) and 0.38% bis (w/v) was prepared. To polymerize the gels, 33 µl NNN/N/-Tetramethylethylene diamine and 2.5 ammonium persulfate (w/w) were added per gram of acrylamide. Gels were polymerized in 0.5 x 12 cm glass tubes for 30 minutes at room

temperature and pre-run at 5 mA per gel for 1 hour. Samples were mixed with an equal volume of 40% sucrose and 0.01% bromphenol blue and layered on the gel. Electrophoresis was conducted at 5 mA/gel for 30 minutes. The final buffer concentration was: Tris, 0.04 M; sodium acetate, 0.02 M; EDTA, 2 mM; acetic acid was used to adjust the pH to 7.8.

Staining

A stock staining solution of 0.1% Stains-All in 100% formamide was diluted to 0.01% with 100% formamide and then further diluted to 0.005% with water to give a working solution having 50% formamide. The gels were stained overnight in the dark at room temperature and destained in the dark in running water for 4 hours. Because of the light sensitivity of stains all, all solutions were stored in the dark.

Scintillation Counting

Radioactivity was quantitated in a Packard Tri-Carb spectrometer. Samples of ^{125}I in 1 ml of solution were added to 10 ml of scintillation fluid containing 66.7% of toluene (w/v), 33.3% of Triton X-100 (w/v), 0.01% 1,4-bis [2-(4-methyl-5-phenyloxazolyl)] benzene (dimthyl POPOP) (w/v) and 0.55% 2,5 diphenyloxazole (PPO) (w/v), and counted with a gain of 40% and a window of 50 to 1000 with an efficiency of approximately 80%.

Photo Microscopy

Photomicroscopy was performed with an A. O. Spencer phase microscope equiped with a Kodak Colorsnap 35 camera. The photomicrographs were taken with Kodachrome II professional color film at shutter speeds of 6 to 12 seconds with normal viewing illumination.

RESULTS

Uptake of [^{125}I] GAT into Peritoneal Cells

The peritoneal cells employed in the first experiment contained 80-90% red blood cells. A preliminary experiment was performed to assess the contribution of red blood cells to the uptake of [^{125}I] GAT by white cells. Peritoneal cells in culture were monitored for 18 hours. During this period the number of red blood cells gradually declined with only a 10% decrease in the white cell population.

Cells from the peritoneal cavity enriched in macrophages were incubated in the presence of 0.15 µg [^{125}I] GAT/ml with a specific activity of 6.6 x 10^5 cpm/µg in different concentrations of fetal calf serum to determine if the serum had an effect on polymer uptake. Each culture tube initially contained approximately 10^5 cells. Approximately 1.5 x 10^4 cells were white and 90% of those were macrophages. Red blood cells constituted 85% of the cells. To determine whether contaminating red blood cells incorporated a significant amount of polymer, parallel sets of cultures were prepared containing greater than 99% red blood cells (1.5 x 10^6 cells/ml). The red blood cells

were cultured with medium containing 10% (v/v) fetal calf serum or no fetal calf serum.

At the time of maximum uptake, the enriched white cell population in control cultures (no serum) contained 0.5% of the label; those in 5% serum, 2.85%; and those 10% serum, 2.92%. Red blood cells in control cultures took up 0.57% of the polymer; those in 10% serum, 1.41% (Figure 4). All of the culture reached a maximum rate in about 6 hours. While the cells in 10% serum leveled off after 6 hours, those in 5% serum and no serum continued to increase for 24 hours.

By evaluating red blood cell contamination of macrophages, the contribution to the maximum uptake was estimated to be 1.5%. Fetal calf serum stimulated the uptake of [^{125}I] GAT by both red blood cells and white cells.

Polymer Stability in Cell Culture

To determine whether fetal calf serum had an effect on [^{125}I] GAT stability in culture, the incubation media from uptake experiments described above was fractionated on a Bio Gel P-30 column. The tissue culture media contained phenol red which eluted in Fractions 9 to 12 (Figure 5) and was used as a low molecular weight marker. Degradation was assessed by the percentage of ^{125}I eluted in fractions 9 to 14. Sixteen percent of the

Figure 4. Uptake of [^{125}I] GAT in red blood cells and white cells. Peritoneal cells containing 1.5 x 10^4 white cells/ml and purified red blood cells containing 1.5 x 10^6 cells/ml were incubated under standard conditions in the presence of [^{125}I] GAT at a concentration of 4.6 x 10^4 cpm/ml with a specific activity of 6.6 x 10^5 cpm/μg for polymer uptake at various serum concentrations. (----) red blood cells, (———) white blood cells.

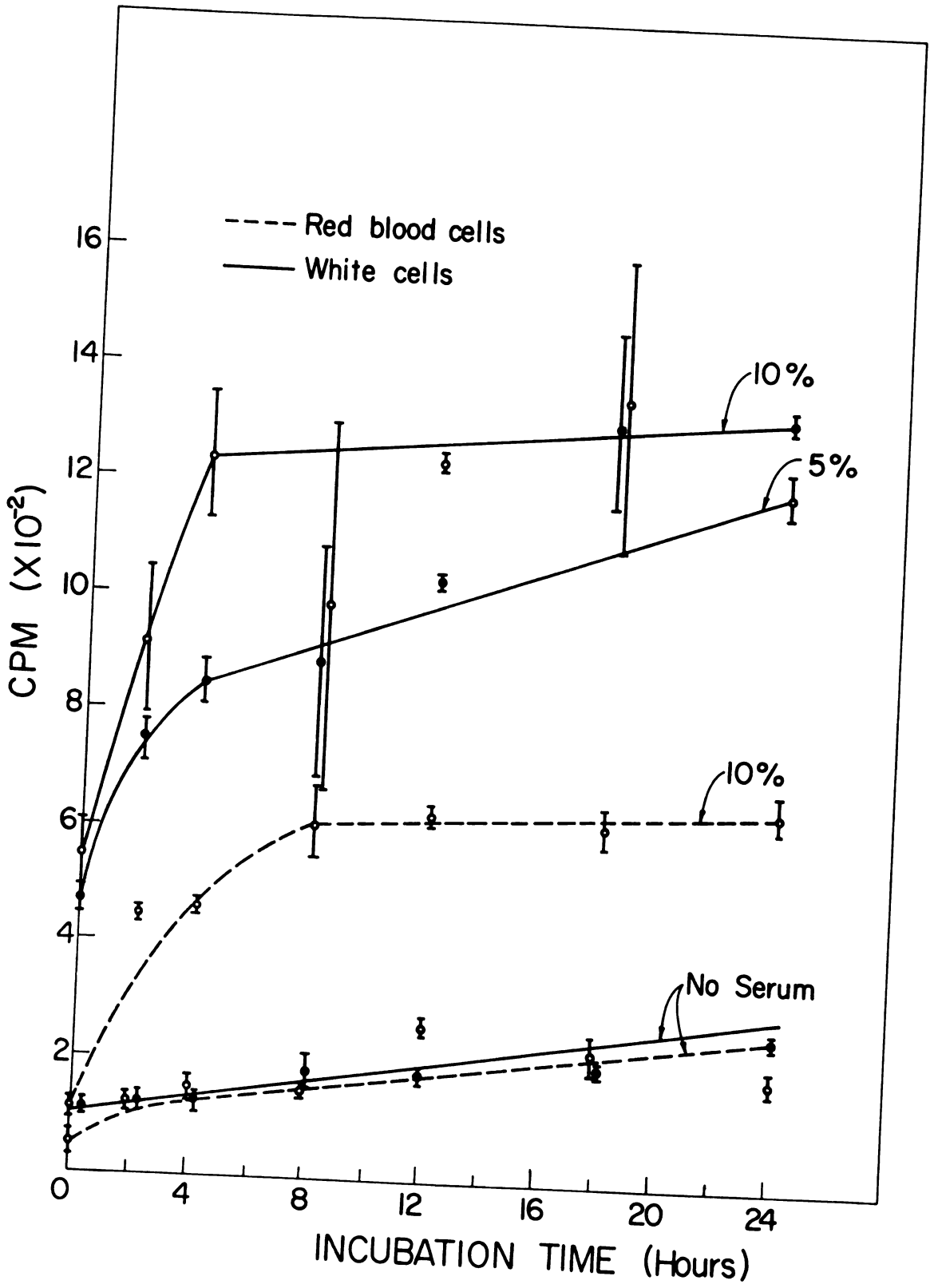
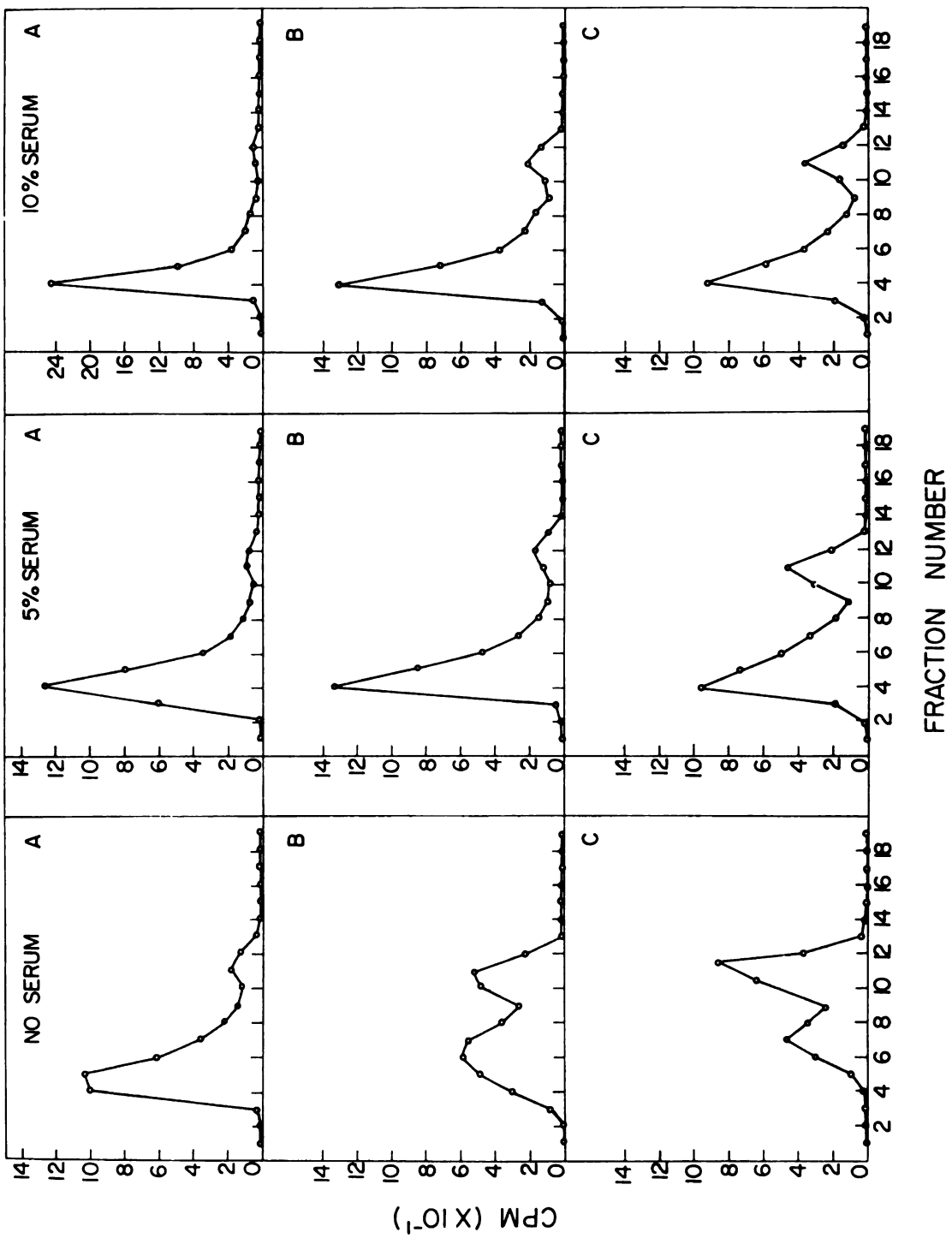

Red blood cells
White cells
10%
5%
10%
No Serum
CPM (X10⁻²)
INCUBATION TIME (Hours)
0
4
8
12
16
20
24
2
4
6
8
10
12
14
16

Figure 5. [^{125}I] GAT stability in cell culture medium containing different concentrations of serum. The sterile conditioned culture media containing no serum, or 5 or 10% serum were incubated with 3.6×10^4 cpm/ml of [^{125}I] GAT with a specific activity of 6.6×10^5 cpm/µg. After 4 hours (A), 12 hours (B), and 24 hours (C), the media were fractionated on a 1 x 32 cm Bio Gel P-30 columns and collected in 50-drop fractions.



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polymer in incubation medium without serum was degraded by 4 hours; 39% was degraded by 12 hours; and 62% was degraded by 24 hours (Figure 5). Polymer in medium containing 5% serum was 8.7% degraded by 4 hours; 14.9% was degraded by 12 hours; and 28% was degraded by 24 hours (Figure 5). [^{125}I] GAT in medium containing 10% serum was 7.7% degraded in 4 hours, 16% degraded in 12 hours and 26.4% degraded in 24 hours (Figure 5). This indicated that fetal calf serum protected [^{125}I] GAT from degradation.

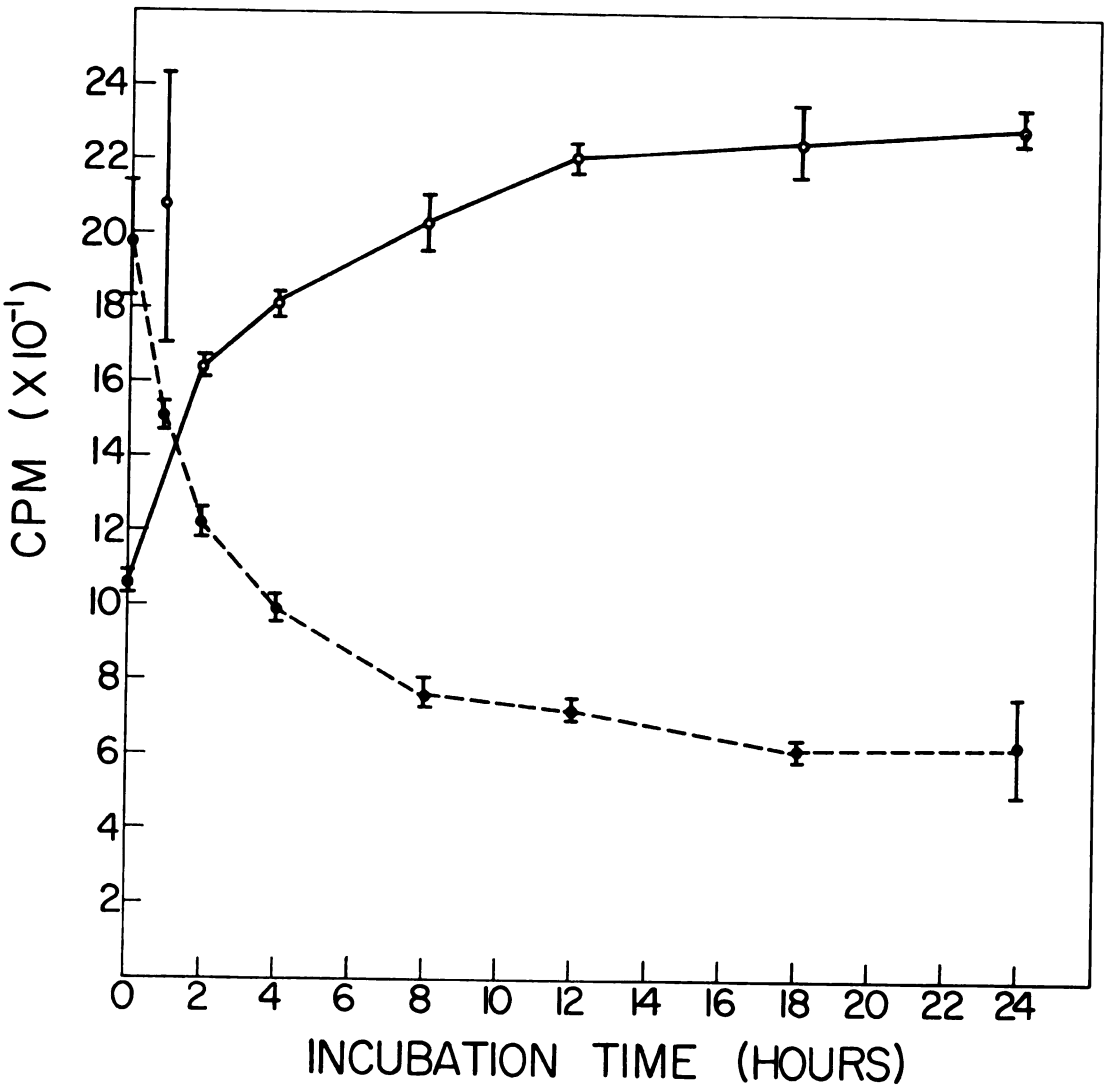
An experiment was also conducted to determine whether the cells were actively metabolizing the polymer and secreting low molecular weight peptides or whether proteases released by necrotic cells were degrading the polymer. 4×10^6 cells (10% white cells) were incubated in 3 ml of medium containing 5% serum for 24 hours. The cells were then removed and [^{125}I] GAT added to the incubation medium ("conditioned medium"). The polymer was incubated under standard conditions for 24 hours and then 1 ml of the medium was fractionated on a column. After incubation in conditioned medium for 24 hours, 32% of the polymer was degraded. Therefore, most of the ^{125}I GAT taken up by the cells in 24 hours was in a polymer form with a molecular weight of at least 30,000 daltons.

Retention of [^{125}I] GAT

2.6 x 10^7 peritoneal cells (20% white cells) were incubated with 1.73 x 10^5 cpm/ml of [^{125}I] GAT at 8.9 x 10^4 cpm/µg for 12 hours, the label was removed, new unlabelled medium added and the cells were monitored for ^{125}I retention. By 12 hours, approximately 59% of the label in the medium was taken up by the cells. A gradual release of radioactivity into the culture medium and a corresponding decline in cellular label was noted (Figure 6). At the end of 24 hours, 36% of the [^{125}I] was still retained by cells.

The stability of the cell population during the incubation was evaluated to determine if the release of label was due to lysed white cells. Under incubation conditions lacking serum, the total cell population was reduced by 43% and the white cell population by 45%. During the 24 hours' incubation in unlabelled medium the total cell population decreased by 72% and of this the decline was 69% in the first 12 hours. The percentage of white cells in the total cell population increased from 19% at 12 hours to 47% at 36 hours with a decrease in total white cells of 25%. The surviving white cells were greater than 99% macrophages. To assess the reliability of the hemacytometer in giving consistent cell counts, four different cultures of the same dilution were counted

Figure 6. Retention of [^{125}I] in cells prelabelled with [^{125}I] GAT. Three ml of peritoneal cells at a concentration of 8.5 x 10^6 cell/ml (20% white cells) was incubated with 2 ml of [^{125}I] GAT at a final concentration of 1.73 x 10^5 cpm/ml (specific activity of 8.9 x 10^4 cpm/mg) for 12 hours. The labelled medium was removed and fresh unlabelled medium was added. At intervals, the medium was removed and the amount of [^{125}I] in cells and medium was determined. (----) cells, (———) supernatant.



with
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GAT
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or 12
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and the variation determined. Cells were counted in the 4×10^4 to 1.13×10^5 cells/ml range with a reliability of 90%.

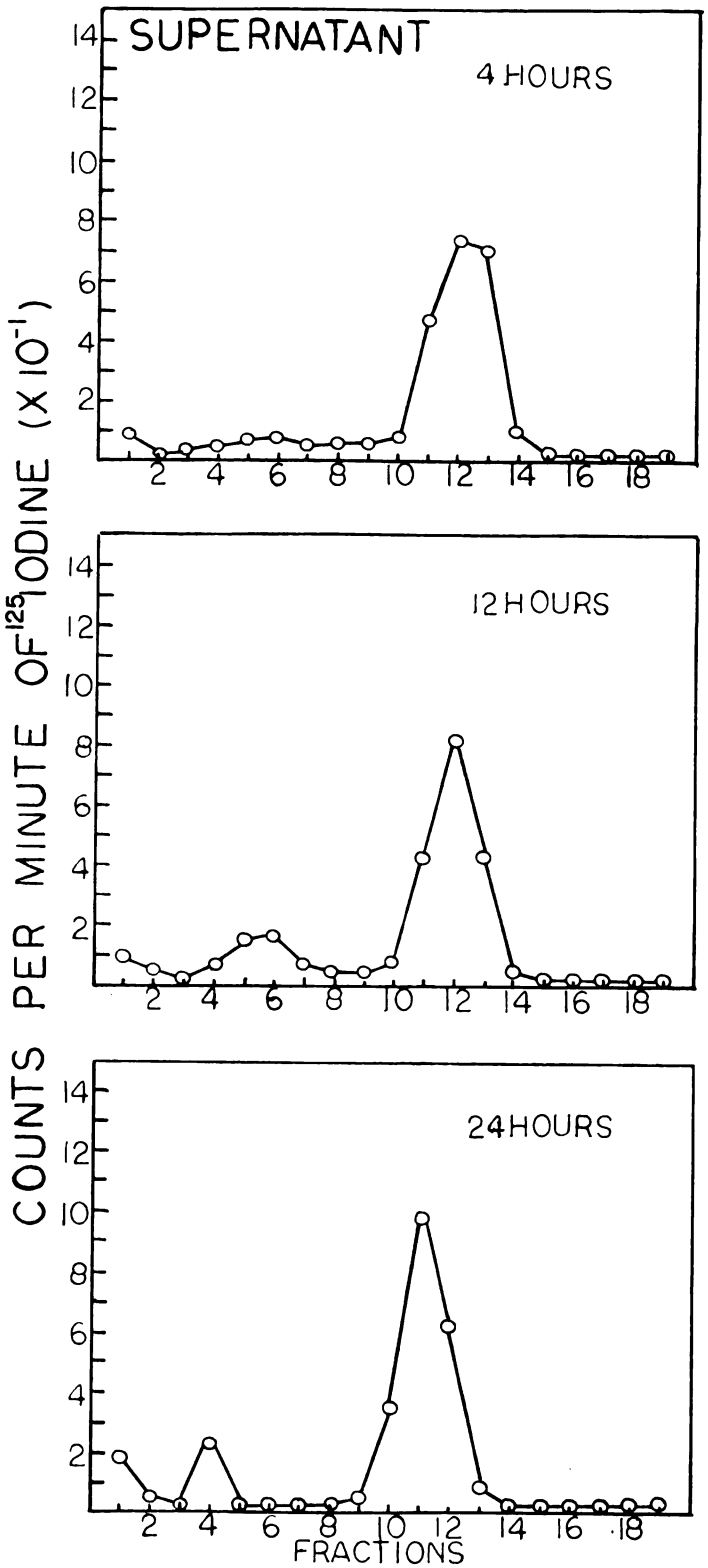
Size Distribution of [^{125}I] GAT Released from Prelabelled Cells

The incubation medium from the retention study was analyzed on a Bio Gel P-30 column to evaluate the molecular weight distribution of the labelled material released into the medium by the prelabelled cells. Fractionation of the [^{125}I] in the initial 12 hours' pulse medium revealed that 88% of the label eluted in the low molecular weight fractions (9-14) (Figure 7). Four hours after the labelled polymer was removed, 88% of the label in the medium was in the low molecular weight range. However, after 12 hours the low molecular weight components accounted for 79% of the label in the medium. Although only 13% of the radioactivity at 24 hours eluted in the high molecular weight range, 76% of this material eluted as a single peak with a molecular weight of at least 30,000 daltons. This indicated that peritoneal cells can secret ingested [^{125}I] in a high molecular weight form.

RNA Extraction

Pancreas was chosen as a model tissue for the development of an RNA extraction procedure because the tissue is known to synthesize ribonuclease, the greatest

Figure 7. Molecular weight distribution of [^{125}I] released into the culture medium by prelabelled cells. Peritoneal cells were treated as described in Figure 6. At interval of 4, 12 and 24 hours, the cells were removed; the culture media were fractionated on 1 x 32 cm Bio Gel P-30 columns; 50-drop fractions were collected.


SUPERNATANT
4 HOURS
12 HOURS
24 HOURS
COUNTS PER MINUTE OF ^{125}IODINE (X 10^{-1})
FRACTIONS
2
4
6
8
10
12
14
16
18

deterrent in the isolation of intact RNA. Degradation was assessed by evaluating ribosomal RNA separated by gel electrophoresis. Initially, the tissue was homogenized in acetate buffer and PVS and then extracted with SDS and phenol. Samples for electrophoresis were taken after the first, second, third and fourth phenol extraction and the ether extraction of phenol. Gel electrophoresis revealed that the RNA was degraded by the end of the first phenol extraction. The procedure was modified in that frozen tissue was homogenized in buffer, inhibitor, SDS and phenol at 0°C. The spectra of pancreatic RNA isolated by the two different procedures were similar (Table 4). Ribonucleic acid from spleen, liver, brain and lung was extracted by the modified procedure and compared to pancreatic RNA isolated by both procedures (Table 4). Pancreatic RNA extracted by the modified procedure was degraded more than RNA from other tissues, but not completely degraded as in the original procedure. No degradation was evident for the other tissues as judged by smearing of bands on a agarose acrylamide composite gel. The 260/280 ratios varied from 1.8 to 2.0. With this procedure undegraded RNA of high purity was extracted from pancreas, lung, liver and brain. This extraction procedure was used in all subsequent experiments.

Table 4. Summary of RNA extracted from different tissues. Ribonucleic acid was extracted from various tissues by the modified procedure and assessed for purity.

Tissue	260/280	280/260
Brain	1.82	0.55
Lung	2.03	0.493
Spleen	1.89	0.53
Liver	1.87	0.54
Pancreas*	1.88	0.53
Pancreas	2.03	0.493

*Isolated with original procedure which involved homogenization and phenol extraction as two separate steps. The modified procedure combined these two manipulations into a single step.

TEAE Cellulose Chromatography of Low Molecular Weight Nucleic Acids

Conditions were established for [^{125}I] GAT uptake by peritoneal cells and a procedure was developed for isolating undegraded RNA. Because such a high percentage of [^{125}I] was retained by peritoneal cells, an effort was made to determine whether the retained label was in a low molecular weight antigen-RNA complex as reported by Gottlieb (47). To do this, TEAE cellulose and slab gel electrophoresis were used to analyze low molecular weight RNA.

The resolving power of TEAE cellulose column was determined by separating adenosine monophosphate from E. coli transfer RNA with a sodium chloride gradient. Adenosine monophosphate was eluted with 0.088 M NaCl and tRNA was eluted with 0.345 M NaCl.

RNA extracted from lung and spleen was assessed for purity (Table 4) and fractionated on TEAE cellulose. The elution pattern of lung low molecular weight components revealed a series of peaks in the region of nucleotides and a single peak which appears to contain several components in the region of tRNA (Figure 8). Chromatography of spleen RNA gave peaks corresponding to nucleotides and tRNA (Figure 9). The peak in the tRNA region constituted 15.1% of the spleen RNA and 7.5% of lung RNA. RNA extracted from 2.8×10^7 peritoneal cells yielded 46.4 OD units with a 260/280 of 1.50. The extracted RNA was fractionated on a TEAE cellulose column; 25% of the RNA eluted in the region of tRNA.

Gel Electrophoresis of Chromatography Fractions

To further characterize the low molecular weight RNA, fractions from the TEAE cellulose column were precipitated with two volumes of ethanol and separated on acrylamide gels. Lung low molecular weight RNA was fractionated on a 7.5% acrylamide gel (Figure 10). The initial fractions of the peak (Fractions 59-62) were resolved into six

Figure 8. Separation of lung low molecular weight RNA by TEAE cellulose chromatography. Lung RNA (45.2 OD_{260} units) was added to a 1 x 24 cm TEAE cellulose column and eluted at 4°C, with a sodium chloride gradient. Fifty-drop fractions in 0.02 M Tris-Cl, pH 8.2 were collected.

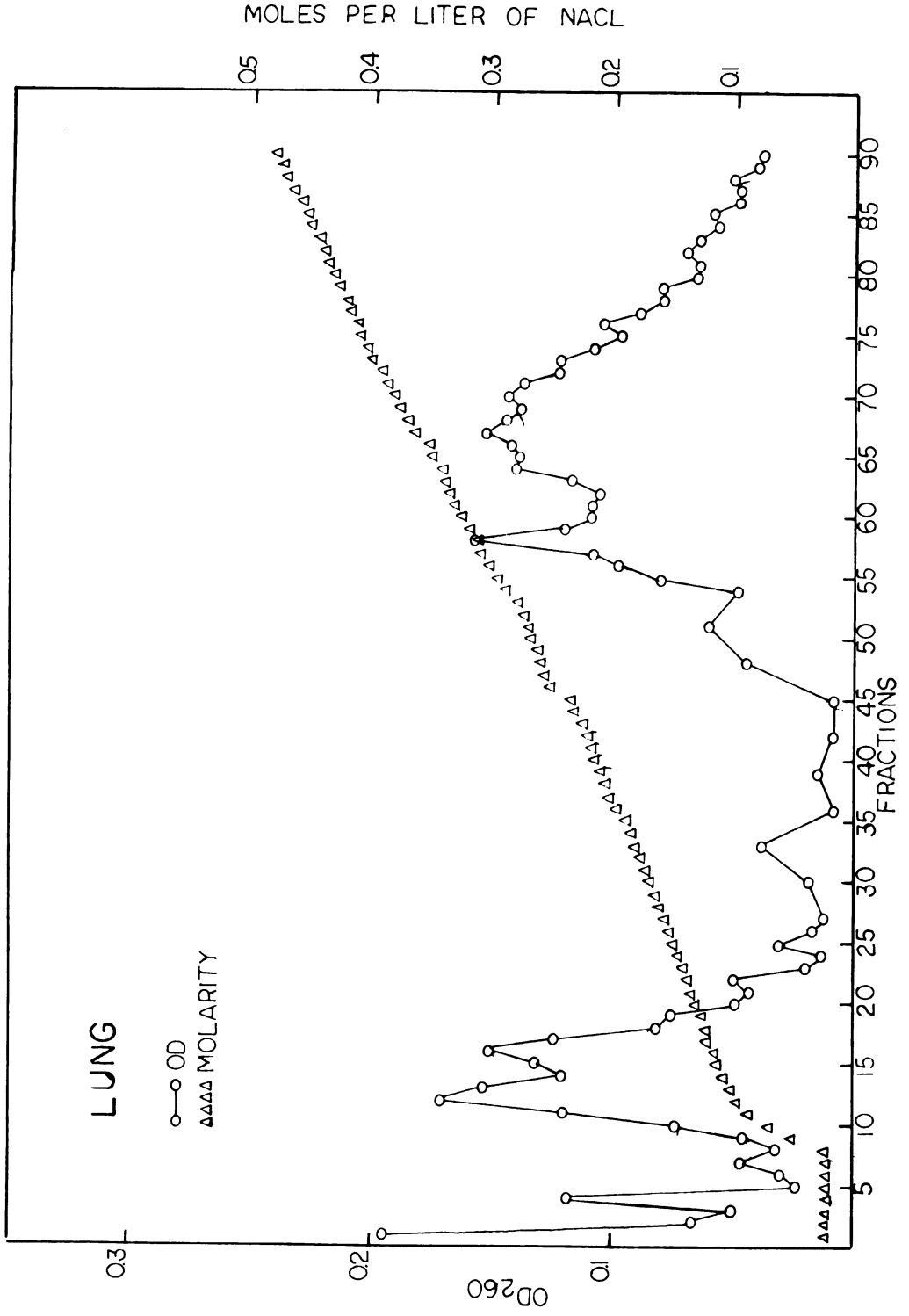

MOLES PER LITER OF NACL
0.5
0.4
0.3
0.2
0.1
LUNG
OD
MOLARITY
OD260
0.3
0.2
0.1
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
80
85
90
FRACTIONS

Figure 9. Separation of spleen low molecular weight RNA by TEAE cellulose chromatography. Spleen RNA (47 OD_{260} units) was added to a 1 x 24 cm TEAE cellulose column and eluted in 50-drop fractions as described in Figure 8.

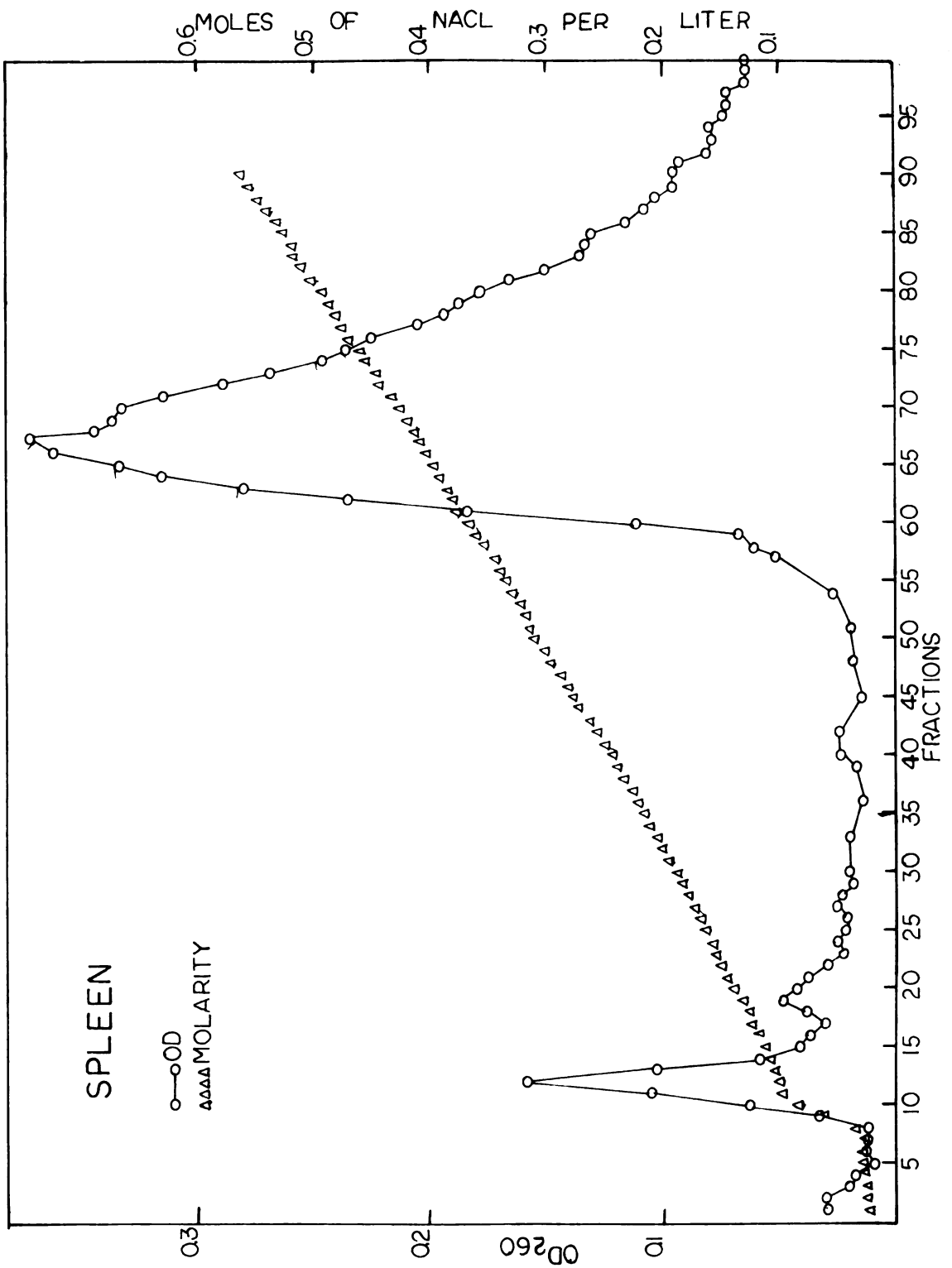
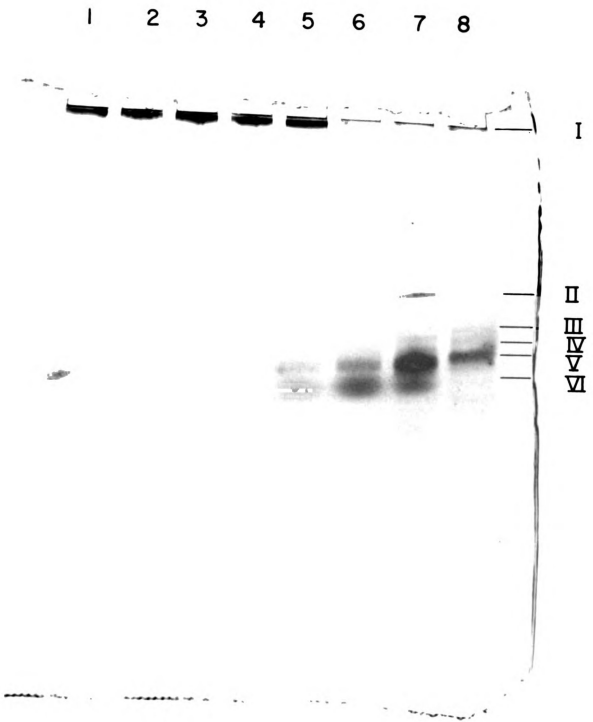

SPLEEN
OD
MOLARITY
OD260
MOLES OF NACL PER LITER
FRACTIONS

Figure 10. Electrophoretic analysis of lung low molecular weight RNA. Electrophoresis was performed on a 7.5% acrylamide slab gel for 3 hours at 4°C and 200 v with TEAE cellulose fractions of lung RNA described in Figure 10. Slot 1 contained fraction 76; slot 2, fraction 73; slot 3, fraction 70; slot 4, fraction 67; slot 5, fraction 64; slot 6, fraction 61; slot 7, fraction 58; and slot 8, 0.4 OD_{260} units of E. coli tRNA.


1
2
3
4
5
6
7
8
I
II
III
IV
V
VI

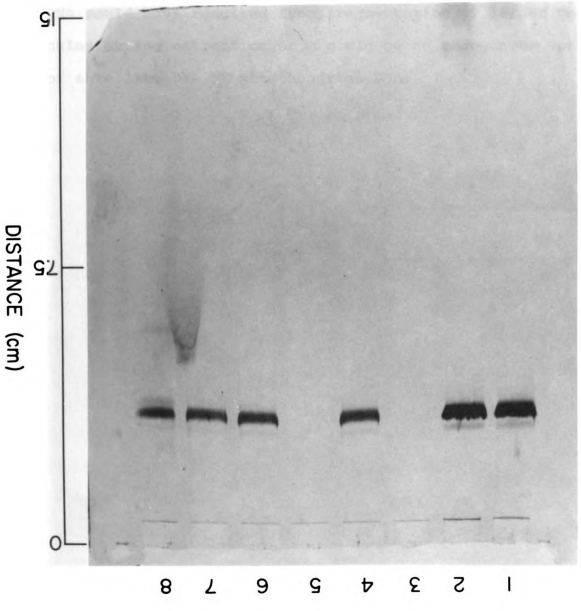
distinct bands. The first five of the six bands comigrated with bands in the E. coli tRNA marker. The elution sequence was bands II, III, IV and V first, band VI second and band I third. Bands VI and I were present in all fractions analyzed. Macrophage low molecular weight RNA resolved on a 7.5% disc gel gave only bands I and VI (Figure 11). This suggested that macrophages contain no unique species of low molecular weight RNA.

Partial Characterization of Band I

Spleen low molecular weight RNA was resolved on a 5% acrylamide gel from TEAE cellulose fractions 55 through 90 of Figure 11. Again, the initial fractions contained tRNA but band I was proportionately greater. In the latter fractions band I was the sole component. To determine the nature of band I, fractions 71 to 81 were electrophoretically analyzed in the presence of pronase, ribonuclease and deoxyribonuclease on an acrylamide agarose composite gel (Figure 12). Pronase was used to identify protein and also to remove any protein which might be associated with RNA and preventing nuclease degradation. Each fraction was ethanol precipitated, dried and redissolved in 100 ml of water. Five µg of the appropriate enzyme was added and the solution was incubated at 0°C for 10 minutes before applying the sample on the gel. Band I was conspicuously absent in slots containing

Figure 11. Electrophoretic analysis of macrophage low molecular weight RNA. Electrophoresis was performed on a 7.5% cylindrical gel for 30 minutes at 5 mA/gel as described in METHODS. Fractions 44, 45 and 46 were on gel (A); fractions 49, 50 and 51 were on (B); 0.5 OD_{260} units of E. coli: tRNA were on gel (C).

Figure 12. Partial characterization of band I. Electrophoresis was performed on an acrylamide agarose composite gel for 2.5 hours at 4°C and 200 v with spleen TEAE cellulose fractions and various degradative enzymes. Slot 1 contained 0.6 OD_{260} units of total spleen RNA and ribonuclease; slot 2, 0.6 OD_{260} units of total spleen RNA and deoxyribonuclease; slot 3, fraction 81 and deoxyribonuclease; slot 4, fraction 79 and ribonuclease; slot 5 fraction 77, pronase and deoxyribonuclease; slot 6 and slot 8, 0.6 OD_{260} units of total spleen RNA.


DISTANCE (cm)
15
7.5
0
1
2
3
4
5
6
7
8

deoxyribonuclease and pronace and deoxyribonuclease alone. Degradation was also evident in slots having whole spleen RNA preparation and deoxyribonuclease. This established Band I to be low molecular weight DNA. Such a species of DNA could have resulted from fragmentation of larger molecules during extraction or it could be an endogenous species of satellite DNA or mitochondrial DNA.

DISCUSSION

Many of the characteristics essential to a cellular defense system were inherent in splenocytes maintained *in vitro*. Such a system would have to be rugged enough to survive the onslaught of microbial invasion. The cells of this system would have to accommodate environmental modulations with a minimum loss of efficiency. Metabolic activities would have to be modulated with the external milieu and the nature of the invader. Lymphocytes and macrophages were cultured for periods up to two weeks with no visible evidence of necrotic cells, whereas the *in vitro* environment destroyed other white cells in a day or two. During incubation, monocytes formed macrophages and giant cells and small lymphocytes enlarged. These transformations also occur *in vivo* with a corresponding increase in the ability of lymphocytes to produce antibody and monocytes to phagocytize particles.

Macrophage-like cells from rat spleen sedimented at the interphase of 27% BSA. After incubation, these cells did not band at this interphase. However, many of the cultured splenocytes had the morphology of macrophages. This suggests that the cells banding in BSA were monocytes and not the larger and more active macrophage of the next

differentiated state in agreement with Bennett and Cohn (26). The failure to detect cells of similar density in brain, liver, and lung is evidence against the existence of monocytes in these tissues. However, this does not negate the possibility that a pre or post monocyte cell type exists in these tissue and that these cells differentiate into the Kupffer cells of liver, the microglial cell of brain and the alveolar macrophage of lung. The lung possesses septal cells, which banded a 23% BSA in association with lymphocytes. This is significant because some histologists believe that septal cells can differentiate into alveolar macrophage.

The white cells induced in the peritoneal cavity were consistently greater than 90% macrophage. In medium containing 10% serum, [^{125}I] GAT uptake by red blood cells was only 50% of the maximum uptake achieved by macrophages (4.2×10^{-4} cpm/cell). Macrophages took up 9×10^{-3} cpm/cell or 5.77×10^{-3} ng/cell, a 200-fold increase over the red blood cell uptake. Since the reliability of the cell count is 90%, a 1% or 2% contamination of red blood cells by macrophages would not have been detected. Therefore, the contribution to uptake by red blood cells is negligible.

Fetal calf serum stimulated [^{125}I] GAT uptake in cultured macrophages. The addition of 10% fetal calf serum caused a 5-fold increase in the uptake of polymer by

macrophages. Macrophages cultured in 5% serum required a longer incubation to achieve a maximum uptake and were incapable of sustaining such a level once achieved. This effect can be partially attributed to protection of the labelled polymer from protease degradation. However, limited [^{125}I] GAT degradation is not sufficient to explain the differences which occurred at zero time. Although at this time the concentration of intact polymer was approximately the same in all cultures, uptake was greatest in cultures containing 10% serum. Even though the polymer in both 5% and 10% serum was protected to the same extent, cells in 10% serum still took up polymer at a much faster rate. Stimulation of uptake can best be explained by the presence of a single serum factor or a series of serum factors which enhances pinocytosis. Such a factor could originate from epigenetic regulation in the developing calf or could merely be metabolic cofactor such as vitamins or coenzymes.

The retention of such a high percentage of polymer in macrophages in the presence of 10% serum implies a specific role for the GAT taken up. Cells involved in nonspecific pinocytosis and general metabolism would rapidly equilibrate with the protein in the external environment. Macrophages could contain an enzyme system capable of utilizing iodinated tyrosine and free iodine as in the pituitary gland. Conceivably, the polymer retained

by the cells is in a processed form capable of invoking an antibody response from lymphocytes. A processed antigen could exist as a free protein or in conjunction with RNA. Regardless of the function, labelled iodine is released into the medium in a form having a molecular weight of at least 30,000 daltons.

Gottlieb stated that immunogenic RNA constituted 4% of total macrophage RNA and that it eluted from a TEAE cellulose column with 0.32 M sodium chloride (46). Since examination of those fractions by gel electrophoresis revealed no unique species of RNA in spleen or macrophages, the antigen-RNA complex either comprises less than 4% of macrophage RNA or it elutes at a different salt molarity. Characterization of slower eluting nucleic acid suggests that the antigen-RNA complex must have a greater affinity for TEAE cellulose than tRNA because the elution profiles had no major ultraviolet radiation absorbing component in the region between nucleotides and the major peak. However, it is possible that immunogenic RNA migrates with tRNA and constitutes a major portion of the difference in the percentage of low molecular weight nucleic acid observed in macrophage and lung total RNA. In this case, identification can only be accomplished by using a radioactive antigen marker. Once the labelled complex is identified, further characterization could be accomplished by degrading the RNA or protein and investigating the nature of the linkage.

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PART II

INTRODUCTION

RNA Synthesis in Mammalian Cells

Ribosomal RNA

Ribosomal RNA (rRNA) is mainly composed of three RNA species and comprises over 80% of total cellular RNA. The three rRNA species are classified according to Svedberg units (S) and are designated 28S, 18S and 5S. The initial recognizable precursor of the 28S and 18S components, 45S, was first observed during studies of rapidly sedimenting nucleolar RNA (8, 9, 10). Confirmation of these species was made using polyacrylamide gel electrophoresis (11, 12).

Because of the slow equilibration of nucleoside pools (66, 67) the typical "pulse chase" experiments were ineffective and precursor studies were conducted using actinomycin D. The inhibitor was administered after brief exposure to labelled uridine to inhibit further isotope incorporation and to allow the processing of precursors to be traced. Labelling kinetics in rat liver suggested a half-life of 6 to 8 minutes for the 45S precursor (9). After a partial hepatectomy total RNA synthesis in regenerating rat livers increased 5-fold (13) and the half-life

of 45S rRNA was reduced to 3.7 minutes (14). The next probable step in the synthesis of 28S and 18S rRNA is the cleavage of 45S RNA to 41S and then 36S RNA (11). The next major precursor is a 32S molecule which is converted to the 28S species found in cytoplasmic ribosomes. This path is 45S → 41S → 36S → 32S → 28S. Eighteen-S rRNA is also synthesized from the 45S presursor via a 20S or 23S intermediate (11, 15): 45S → 23S → 18S. Only 50 to 60% of the 45S molecule is used to make 18S and 28S RNA (16, 17). The only acceptor of methyl groups is the 45S rRNA precursor, and all methyl groups are conserved in further processing (11, 18).

The 5S molecule is found in the nucleolar particle with the 45S species (19), but the 5S species does not hybridize with other rRNA molecules (20) and is apparently made from different precursors. In addition, the 5S molecule must pass through a 5S pool before entering the ribosomes (21).

Transfer RNA

Transfer RNA (tRNA) is the next most abundant RNA species in mammalian tissue. In polyacrylamide gels, tRNA migrates more rapidly than ribosomal 5S RNA. Recent investigations have established the existence of a tRNA precursor. After a 30 minute labelling period in HeLa cells, a radioactive peak was observed in the region of

tRNA and 5S rRNA distinct from tRNA and 5S RNA (22, 23). At later times the peak gradually disappears and a new radioactive peak comigrates with the 4S peak of tRNA. In vitro conversion of earlier labelled species to tRNA has been accomplished (24). The slower migration of the pre-tRNA molecule is due to a longer nucleotide sequence and not merely a conformational structure different from tRNA (24). Pre-tRNA is deficient in methyl groups and has a lower content of pseudouridine (25).

Nuclear RNA

Harris noted that the amount of label appearing in the cytoplasm of macrophages and connective tissue was insufficient to account for all the RNA synthesized in the nucleus (26, 27). Short term labelling experiments indicate that the nucleus contains rapidly labelled RNA which sediments throughout a 5% to 20% sucrose gradient and is almost totally degraded without ever entering the cytoplasm (28). Sedimentation values for this heterogenous nuclear RNA (HnRNA) range from 2 to 100S. Although the very large HnRNA molecules have a low guanine and cytosine content as in DNA (1, 29, 30), total nuclear RNA is diverse in base composition (31, 32). The DNA-like base composition of the larger molecules is partially attributed to the presence of polyadenylate segments on the 3' terminus of messenger RNA which is also found in HnRNA

(33, 34, 35, 36). It was postulated that the polyadenylate attachment may be a necessary processing step which stabilizes the messenger RNA and facilitates transport into the cytoplasm (37), but the conspicuous absence of the polyadenylate segment on histone messenger RNA makes such an encompassing hypothesis unlikely (38). This concept is further abated by the occurrence of messenger RNA in protein complexes which are also postulated as the form of the processed messenger (39, 40, 41, 42). Messenger RNA sediments at approximately 10S (43, 44) and can be extracted from the nucleus or removed from cytoplasmic polysomes.

Control of the Formation of RNA Precursors

RNA precursor formation and cleavage in mammalian cells is a process which normally occurs in minutes and occupies a small fraction of the cellular generation time. By utilizing suboptimal cell culture conditions and inhibitory drugs, precursor processing can be manipulated to allow each cleavage step to be thoroughly evaluated. Cycloheximide at a concentration which inhibits 99% of protein synthesis reduces RNA synthesis and causes the accumulation of rRNA precursors. But the drug fails to block precursor processing and ribosome assembly completely (45). Puromycin also reduces RNA synthesis while allowing 45S precursor and methylation to occur, but unlike cycloheximide, ribosome formation is completely blocked in that 28S

rRNA is made and 18S rRNA is not. Once the drug is removed normal processing continues. Puromycin and cycloheximide together allow reduced ribosome formation (46). Methionine deprivation to an extent which inhibits 70 to 80% of protein synthesis allows precursor formation but inhibits 28S and 18S rRNA formation by reducing methylation of 45S RNA by 80% and 32S by 60%; valine deprivation to the same extent permits reduces synthesis of 28S and 18S RNA molecules (47). Incubation of HeLa cells at reduced temperatures stops rRNA processing. Below 20°C, no 45S RNA is formed; at 15°C conversion of 45S to 32S is completely inhibited and at 25°C, 32S cleavage to 28S is stopped (48). Prolonged incubation at low temperatures causes the transient 41S intermediate to accumulate. Transfer RNA precursor can be isolated as a distinct peak in cells that have been methionine starved (49) or maintained at a suboptimal temperature (50).

RNA Synthesis During Development

By far the most intensive studies of RNA synthesis during development system have been the investigation of oogenesis in echinoderms and amphibians. In both systems the small immature oocytes which occur before yolk disposition or during the previtellogenesis stage are active in RNA synthesis and are responsible for RNA accumulation; the late mature oocytes are relatively quiescent (51, 52).

In Xenopus laevis oocytes at all stages of development can be found maturing simultaneously in the ovary. Duryee classified the maturing oocytes into distinct numerical stages with each having its own chromosomal physiology and nuclear structure (53). Stage 3 constitutes the early lampbrush stage which marks the appearance of lampbrush chromosomes. Stage 4 is the maximum lampbrush period. According to the results obtained by Davidson (54), the stage 3 oocyte possesses about 20% of the RNA contained in the mature stage 6 oocyte. The total RNA increases to the stage 6 value by the end of the maximum lampbrush stage. In Xenopus oocytes at either stage 4 or 6, 95% of the RNA is ribosomal and therefore measurement of total RNA content is essentially a measure of rRNA content. Labelled RNA extracted from lampbrush stage oocytes after a 30 minute exposure to labelled precursor has the base composition and sedimentation characteristics of rRNA. The same is true of RNA extracted from oocytes labelled several hundred times longer. In contrast, a large proportion of the high molecular weight RNA synthesized per unit of time in HeLa or liver cells is nonribosomal (54). After stage 4 no more rRNA is made until the nucleoli appear in the fertilized egg during gastrulation. In the chick embryo new rRNA begins to be made at the mid-cleavage stage (55); mouse embryos, however, are able to synthesize new rRNA soon after fertilization (56).

Brown and Littna noted a sudden burst of informational RNA (mRNA) synthesis in Xenopus toward the end of oogenesis (57, 58). Using its DNA-like base composition as a means of characterization, they estimated that the unfertilized ovulated egg contains at least one nanogram of new mRNA. The total new mRNA in the early Xenopus gastrula containing thousands of active nuclei, is only about 10ng. Consequently, the synthesis of one nanogram of mRNA within the 12 hours of the ovulation period constitutes a tremendous increase in mRNA synthesis. Hormones which induce ovulation are believed to be the cause of the sudden burst of mRNA synthesis. In sea urchin, mRNA synthesis appears to occur at least a month before maturation of the egg. Female sea urchins incubated with labelled RNA precursor three months before analysis has labelled mRNA still present in mature oocytes (59). Synthesis of mRNA continues under stimulatory conditions up to a week before shedding (60). The fact that in both species enucleated and actinomycin D treated fertilized eggs are able to develop to the gastrula stage without synthesizing new RNA indicates the present of maternal "programmed" or "masked" messenger RNA which is somehow activated by fertilization to direct early embryogenesis (61).

Differentiation of the Rat Pancreas

Past studies of the embryonic rat and mouse pancreas have partially defined the transitory stages in the differentiation of the anlage to the mature organ (1, 2, 3, 4, 5). Most of the investigations were concerned with the enzymatic and morphological differences which accompany phenotypic maturation. Therefore, considerable data are available on the synthesis of secretory proteins and organelles and the effects of various metabolites and drugs on their *in vitro* occurrence (6). Currently, Parsa, Marsh and Fitzgerald have observed the methionine dependent differentiation of rat pancreas *in vitro*. Day 13 anlages maintained in methionine deficient medium for nine days were able to grow, but there was only limited morphological differentiation and little increase in the enzymatic activity of amylase, lipase or chymotrypsin, proteins produced specifically by the mature tissue in large amounts. Addition of methionine or S-adenosyl methionine (SAM) resulted in considerable morphological differentiation and increased level of these proteins within 24 hours (62). Acinar cell proliferation required 30 mg/l of methionine and differentiation required 50 mg/l. Because SAM or choline (methyl donors) could substitute for methionine and homocysteine (demethylated methionine) could not, it was concluded that the concentration of

available methyl groups influences the differentiation of pancreas *in vitro* (63). Because of previous studies of methionine deprivation in HeLa cells (47), the most obvious place to investigate an effect is in RNA processing. Unfortunately, few laboratories have attempted to elucidate the role of RNA in the differentiation of the pancreas and those that have were unable to detect any diversity in the RNA synthesized at the different fetal ages (7). Mainly this can be attributed to the small amount of tissue obtainable from rat and mouse embryos; the prodigious concentration of ribonuclease in the more matured pancreatic rudiments; the lack of analytical methods capable of resolving the possible differences which may occur.

Research Approach

As a standard technique for analyzing RNA, polyacrylamide gel electrophoresis is replacing sucrose zonal sedimentation and because of its superior speed and resolution, it removes several of the obstacles confronting pancreatic RNA studies. The technique permits analysis of small samples and provides rapid separation of RNA from the contaminating residual ribonuclease which survives phenol extraction. Polyacrylamide gel electrophoresis has been instrumental in elucidating the discrete steps in the processing of RNA by mammalian tissues.

Using polyacrylamide gel electrophoresis, I have attempted to determine if ribosomal RNA synthesis in the differentiating rat pancreas occurs differentially as in amphibian oogenesis or continuously as in other mammalian systems. By resolving RNA made at a suboptimal pH, I have obtained contrasting profiles of RNA made under conditions of normal and abnormal synthesis. I have devised methods for preparing undegraded RNA from rat pancreas suitable for electrophoretic analysis. The sensitivity of existing methods for gel electrophoresis has been increased. I have defined culture conditions for [^{3}H] uridine incorporation into RNA and evaluated changes in the labelled population of RNA during cytodifferentiation. This study clearly establishes the feasibility of polyacrylamide gel electrophoresis as an analytical tool in the study of RNA in embryonic systems.

METHODS

Rat Breeding

Male and female Sprague Dawley rats were paired in breeding cages for 12 hours. Dropped vaginal plugs marked conception. Approximately 70% of the plugged females were pregnant and litters routinely exceeded 12 rats.

Incubation of Pancreatic Rudiments

Pregnant female rats were sacrificed by cervical dislocation and decapitation. Placentas were aseptically removed and placed in Earle's balanced salt solution. Fetal dissections were performed in Earle's balanced salt solution (EBSS) under a Nikon dissecting microscope. Embryonic pancreases excised and placed in Eagle's minimum essential medium (MEM) containing Hank's balanced salt solution. The incubation conditions were similar to those described by Shaffer (67). The pancreases were then cut into pieces of 5-15 µg and placed on sterile Millipore filters (pore size 0.22µ, 13mm). When the excess media was removed, the tissue became attached to the filter. To study the kinetics of RNA and protein synthesis, six filters each containing approximately 20 µg of tissue were

floated (tissue down) in a 100 x 20 mm Falcon dish containing 7 ml of complete MEM having 5μCi/ml of [^{3}H] uridine (25.7 Ci/mM) or [^{3}H] leucine (23 Ci/mM). For electrophoretic analysis of RNA, four filters each containing approximately a total of 50 to 150 μg of tissue were floated on 7 ml of complete MEM having 5μCi/ml of [^{3}H] uridine. Complete culture medium contained the following components: 87% (V/V) Eagle's MEM, 10% (V/V) fetal calf serum, 100 unit/ml of penicillin and 100 μg/ml of streptomycin, an amino acid supplement was added which increased the final concentration of L-methionine from 0.1 mM to 0.3 mM of L-argonine from 0.6 mM to 1.8 mM, of L-glutamine from 2 mM to 4 mM. The tissue was incubated at 37°C under a humidified atmosphere of 95% air and 5% CO_2.

Uptake Analysis of [^{3}H] Uridine and [^{3}H] Leucine

After various incubation periods, the filters were removed, washed with 1 ml of Eagle's MEM at 4°C and the tissue was scraped off. The pieces of pancreas were placed in a 1 ml tissue grinder (Duall) with 1 ml of acetate EDTA buffer containing 10 mM sodium ethylenediamine tetraacetate (EDTA), 10 mM sodium acetate buffer (pH 5.1) and 5 μg/ml of polyvinyl sulfate (PVS); homogenization was performed by 20 strokes of a teflon motorized pestle at 0°C.

Total Uptake of [^{3}H] Uridine and [^{3}H] Leucine Precursor into Homogenates

To evaluate total uptake of label, 0.2 ml aliquots of homogenate were added to scintillation vials, and solubilized in 0.5 ml of 1 M KOH at 90°C for 1 hour. Ten ml of triton-toluene scintillation fluid (METHODS, part I) were added and the radioactivity was counted in a scintillation counter.

Incorporation of Labelled Precursors into TCA Precipitable Material

0.2ml aliquots of homogenate were precipitated at 0°C for 15 minutes with 10% TCA and 100µg of BSA as a carrier for [^{3}H] leucine labelled protein or 100µg of yeast RNA as carrier for [^{3}H] uridine labelled RNA. The precipitate was collected on a 2.4 cm glass fiber filter (GF/C). Alkaline resistant uridine counts were assessed by hydrolyzing 0.2 ml of homogenate with 0.5 ml of 1 M KOH at 60°C and precipitating in the presence of 100µg of BSA with 10% TCA. The precipitate was collected on filters, and the filters were incubated at 90°C for 1 hour in 0.5 ml of 1 M KOH and counted. Soluble counts were determined by subtracting the TCA precipitable counts from the counts in an equal volume of homogenate. Because TCA causes quenching in our scintillation system, the soluble counts could not be measured directly.

Protein Determination

Two 0.1 ml aliquots of a homogenate were placed in 0.4 ml plastic microfuge tubes (Beckman Instrument Co.) with 0.2 ml of 10% TCA and precipitated at 4°C for 4 hours. The solution was centrifuged for 5 minutes in a Microfuge (Beckman), the supernatant removed and the pellet dried. Protein was estimated according to the method of Lowry (64) as modified by Rutter (65).

Gel Electrophoresis

Slab gel electrophoresis was conducted in a customized Raymond vertical analytical cell according to the methods of Peacock (see Gel Electrophoresis, Part I). The apparatus was made to accommodate gels of 1/16 inch and 1/8 inch thickness with slots for thirteen samples. Because of the limited amount of tissue and the ribonuclease susceptibility of the pancreatic RNA, a method was sought which allowed sensitive analysis of crude RNA extractions. Agarose acrylamide gels of 1/8 inch thickness did not give the sensitivity of the 1/16 inch gel. The 1/16 inch gel was used in later analysis, but because of its fragility, the thinner gel had to be handled with great care.

Gel Formation

The gels used were agarose acrylamide composites with 2% acrylamide and N,N'-ethylenebisacrylamide (bis) and 0.5% agarose. The acrylamide and bis were purchased as Cyanogum 41 in a ratio of 19:1. Two gels 1/16 inch thick and 12 cm long were formed from an 80 ml gel solution of 0.4 g agarose, 54.5 ml of water, 8 ml of 20% Cyanogum 41, 5 ml of 6.4% 3-dimentylaminopropionitrile, 8 ml of 10X Tris EDTA borate buffer and 2.4 ml of 1.6% ammonium persulfate. Gels were polymerized for 1 hour at 25°C and pre-run for 1 hour at 4°C 100v. Extra gels were stored horizontally under buffer at 4°C up to 4 days with no visible distortion.

RNA Extraction

After evaluating various extraction procedures, the following standard procedure was employed. Washed labelled tissue was homogenized with 20 strokes of a 1 ml teflon tissue grinder (Duall) at 0°C in the presence of 47.5 1 of standard RNA extraction buffer (10 mM sodium acetate, pH 5.1, 10 mM sodium EDTA and 5 µg/ml PVS), 2.5 µl of 10% SDS and 50 µl of 88% redistilled phenol. The homogenate was placed in a microfuge tube and chilled for 30 seconds in an ice bath. The aqueous and phenol phases were separated by centrifuging the mixture for 2 minutes in a Beckman Microfuge at 4°C. The aqueous layer was removed and

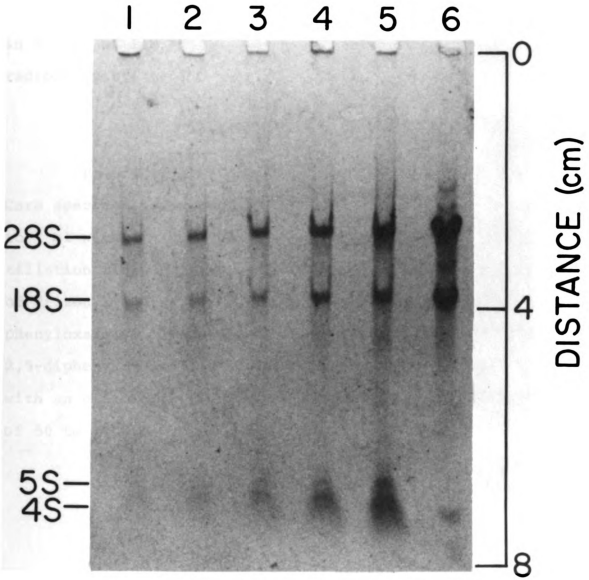
re-extracted in 25 µl of redistilled phenol by mixing with a Micromixer (Beckman) at 4°C. The mixture was chilled and centrifuged, and the aqueous phase was removed and frozen at -20°C.

After two phenol extractions the final volume of aqueous solution ranged from 5 to 20 µl and this was sufficient for direct gel application without further adjustment of the RNA concentration. With this procedure the RNA from 19 µg of 15-day fetal pancreas was sufficient for electrophoretic analysis (Figure 1). The optimal amount of RNA for electrophoretic analysis was 0.1 to 0.5 OD_{260} units.

Electrophoresis Conditions

After the last phenol extraction, between 5 and 15 µl of aqueous phase were rapidly mixed with 5 µl of layering solution at 0°C. The layering solution was 40% sucrose, 0.1 mM EDTA and 0.01% bromphenol blue. The sample was then layered in the gel slots. Gels were run at 4°C and 50 v for 30 minutes to allow the sample to enter the gel and then at 100 v for 2 hours. Staining was conducted overnight in a Stains-All working solution. The gels were scanned in a model 240 Gilford equipped with a Gilford linear scanner and a Sargent recorder.

Figure 1. Photograph of RNA from varying amounts of 15-day pancreas. RNA was extracted in 100 µl total volume as described in METHODS from 15-day pancreas cultured for 6 hours. Slot 1 contained RNA extracted from approximately 19 µg of tissue; slot 2, RNA from approximately 57 µg of tissue; slot 3, RNA from approximately 95 µg of pancreas; slot 4, RNA from approximately 190 µg of tissue; slot 5, RNA from 15-day liver cultured for 6 hours, and alot 6, 0.1 OD_{260} units of hepatoma rRNA.


1
2
3
4
5
6
0
4
8
DISTANCE (cm)
28S
18S
5S
4S

Determination of Radioactivity in Gels

After destaining the gel slots were cut out in strips and sliced into approximately 1 mm pieces with the razor blades spaced 1 mm apart. Each slice was hydrolyzed in 0.5 ml of 1 M KOH at 90°C for 1 hour and analyzed for radioactivity.

Scintillation Counting

Radioactivity was quantitated in a Packard Tri-Carb spectrometer. Alkaline treated samples were brought to 1 ml with distilled water and added to 10 ml of scintillation fluid containing 66.7% of toluene (v/v), 33.3% of Triton X-100 (v/v), 0.01% 1,4-bis [2-(4-methyl-5-phenyloxazoly)] benzene (dimethyl POPOP) (w/v) and 0.55% 2,5-diphenyloxazone (PPO) (w/v). Counting was conducted with an efficiency of 20% at a gain of 60% and a window of 50 to 1000.

RESULTS

Development of RNA Extraction Methods

RNA Extraction from Embryonic and Adult Pancreas

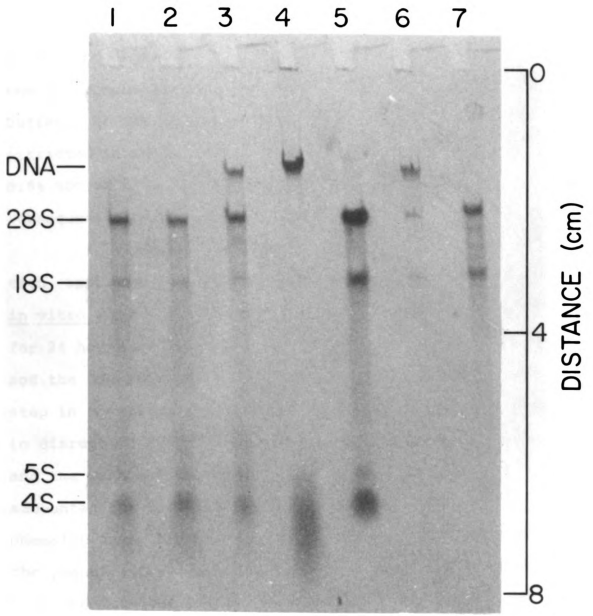
Because of the production and secretion of ribonuclease by rat pancreas, undegraded RNA is very difficult to extract from this tissue. RNA isolation usually entails homogenization in pH 9.0 to 5.1 buffer containing 0.5 to 1% SDS followed by phenol extraction. However, none of the major RNA species in RNA from liver (control) could be detected in electropherogram of RNA prepared according to METHODS, Part I from adult pancreas. To determine exactly where degradation occurred in the isolation process, electrophoresis was conducted on the extraction solution after every manipulation. Previously, control experiments showed that PVS, SDS and phenol had no effect on the migration of hepatoma rRNA on agarose acrylamide gels. Complete degradation of 28S and 18S RNA was evident after the first phenol extraction. This indicated that degradiation had occurred between disruption of the tissue and addition of the phenol and SDS. To shorten this interval, the pancreas was homogenized in a solution of extraction buffer as

above together with an equal volume of 88% phenol. Electrophoretic profiles of this pancreatic RNA were comparable to migration patterns of RNA from liver, lung and spleen.

The pH and extraction buffer composition were varied to determine whether different RNA species could be selectively extracted and if degradation could be reduced. Brawerman, et al. (69) reported that sequential extraction with Tris-Cl at pH 7.6 and 9.0 resulted in extraction of rRNA and selective extraction of polyadenosine enriched RNA. RNA from 17-day fetal rat pancreas extracted in 0.1 M Tris-Cl at pH 7.6 and pH 9.0 and resolved by gel electrophoresis after each extraction yielded only decreasing quantities of rRNA and tRNA.

To further define optimal conditions for RNA extraction, buffer composition, pH, temperature and SDS concentration were varied. Pancreatic rudiment from 17-day fetal rat pancreas was extracted under different conditions and subjected to electrophoresis (Figure 2). Extractions at pH 5.1 in RNA buffer, pH 7.6 in Tris-Cl buffer and pH 9.0 in Tris-Cl yielded increased quantities of DNA and increased RNA degradation. Increasing the SDS concentration to 1% from 0.5% in RNA buffer, pH 5.1, resulted in extraction of more DNA. Reducing the SDS concentration to 0.25% in Tris-Cl, pH 9.0 resulted in extraction of less DNA than in the same buffer with 0.5% SDS. RNA extracted in pH 5.1 RNA extraction buffer and in pH 7.6 Tris-Cl yielded comparable RNA extractions

Figure 2. Electrophoretic analysis of RNA extracted at different pH's, temperatures and SDS concentrations from approximately 360 µg of 17-day pancreas. Slot 1 contained RNA extracted at pH 5.1 in standard RNA extraction buffer (acetate buffer) with 0.5% SDS at 4°C. Slot 2 contained RNA extracted at pH 7.6 in 0.1 M Tris-Cl buffer with 0.5% SDS and 5 µg/ml of PVS at 4°C. Slot 3 contained at pH 9.0 in 0.1 M Tris-Cl with 0.5% SDS and 5 µg/ml of PVS at 4°C. Slot 4 contained RNA extracted by shaking on a micromixer (Beckman) for 30 seconds and incubating at 60°C for 30 seconds over a 4 minute period. The extraction buffer was standard RNA extraction buffer containing 2% SDS and 30 µg/ml of PVS. Slot 5 contained RNA extracted at pH 9.0 in 0.1 M Tris-Cl having a reduced SDS concentration of 0.25%. Slot 6 contained RNA extracted in standard RNA extraction buffer having an increased SDS concentration of 1% SDS. Slot 7 contained 0.1 OD_{260} units of hepatoma rRNA (marker).


1
2
3
4
5
6
7
DNA
28S
18S
5S
4S
0
4
8
DISTANCE (cm)

with slightly more degradation occurring at pH 7.6. RNA extracted at pH 9 in 0.1 M Tris-Cl was more degraded and contained considerably more DNA than RNA extracted at pH 7.6. Elevating the extraction temperature from 4°C to 60°C in pH 5.1 RNA extraction buffer and 1% SDS resulted in extraction of DNA and degraded RNA. More DNA was extracted by increasing the SDS concentration or increasing the pH of the extraction buffer. It was concluded that intact RNA was optimally extracted in pH 5.1 standard RNA extraction buffer and 0.5% SDS at 4°C. These conditions were employed for all subsequent experiments.

To evaluate the efficiency of the micro RNA extraction, approximately 200 µg of 17-day pancreas was labelled in vitro with [^{3}H] uridine at a concentration of 5 µCi/ml for 24 hours as described in METHODS. The tissue was washed and the RNA extracted. Samples were counted after each step in the extraction. The homogenizer and vessel used to disrupt the tissue was washed three times with buffer and the wash solution counted. The pooled solutions accounted for 35.3% of the total [^{3}H] uridine counts. The phenolic phase and the interphase of denatured protein from the phenol extraction contained 23.2% for the first extraction and 7.9% for the second. 33.5% of the total [^{3}H] uridine was in the final RNA solution (9 µl) which would normally have been applied to a gel. Similar percentages were obtained with 14-day and 19-day pancreas. TCA

precipitation of phenol extracted counts yielded the same percentage of counts as was found in the tissue homogenate.

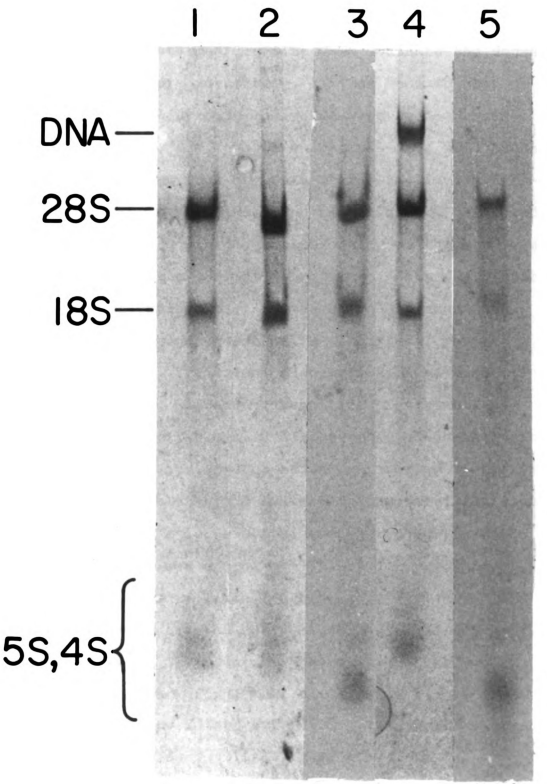
Homogenization of the tissue in the 1 ml homogenizer caused the small volume of rather viscous homogenate to be distributed over a large area of the homogenization vessel. Consequently, a large percentage of uridine counts could not be removed for extraction without using a centrifuge. Centrifugation would have resulted in a longer extraction time and more degradation. So, an increase in extracted RNA counts was comprised for minimum degradation.

Electrophoretic Analysis of RNA from Pancreatic Rudiments During Cytodifferentiation

RNA was extracted from fetal rat pancreas of different embryonic ages to determine whether any major differences in extracted RNA could be detected during differentiation. Figure 3 demonstrates that gels of RNA prepared from 15, 16, 17 and 20-day pancreases gave similar band patterns. The major bands corresponded to 28S and 18S rRNA in hepatoma rRNA; 5S and 4S RNA were detected, but were not well resolved on these gels. This suggested that no difference in the percentage and relative mobility of major RNA species, extracted under standard conditions, occur during pancreatic cytodifferentiation.

An inherent problem in this study was that older rudiments contain more ribonuclease than younger less

Figure 3. A composite photograph of stained gels showing RNA extracted from rat pancreases of different embryonic ages after various periods *in vitro* RNA was extracted in volumes from 100 to 400 µl having 49.5% standard RNA extraction buffer, 0.5% SDS and 50% phenol (88%) and subjected to electropharesis as described in METHODS. The total extraction volume was varied to accommodate the limited amount of tissue obtained from younger embryos. Slot 1 contained RNA from 15-day pancreas cultured for 4 hours; slot 2, RNA from 15-day pancreas cultured for 24 hours; slot 3, RNA from 16-day pancreas cultured for 6 hours; slot 4, RNA from 17-day pancreas cultured for 12 hours, and slot 5, RNA from 20-day pancreas cultured for 6 hours.


1
2
3
4
5
DNA
28S
18S
5S,4S

differentiated rudiments (2). Even mild degradation could decrease the amount of RNA in the major classes and produce species of intermediate size. To evaluate the degree of degradation more accurately, similar stained gels of 16, 17, 19 and 20-day pancreatic RNA were scanned. As shown by Figure 4, the major RNA species--28S, 18S, 5S and 4S--were present at each day, and there was little variation in relative mobilities. The amount of DNA extracted was variable. In addition, occassionally an RNA band was detected between the 18S and 5S regions. This was probably an artifact of extraction. By measuring the area under the major peaks in gel scans of RNA from fetal pancreas at different ages, the extent of degradation between tissues containing different quantities of ribonuclease could be assessed (Table 1). All ages had essentially the same relative amounts of each major RNA species. These were also comparable to RNA extracted from fetal rat liver which was used as a low ribonuclease control. The ratio of 28S to 18S RNA was approximately 2:1 for pancreas of all ambryonic ages in Table 1 except 15 and 20-day. The variation in the ratio of 28S and 18S RNA in 15-day pancreas was due to the small amount of RNA applied to the gel. This caused the background absorbance to be higher and created uncertainty in approximating the peak area. The same situation holds for 20-day pancreatic RNA.

Figure 4. Representative scans of RNA extracted from rat pancreases at different embryonic ages. RNA was extracted and resolved by gel electrophoresis. Gels were stained with Stains-All and scanned at 570 nm as described in METHODS. Gel migration is from left to right; major peaks were identified by the migration of standard hepatoma rRNA.

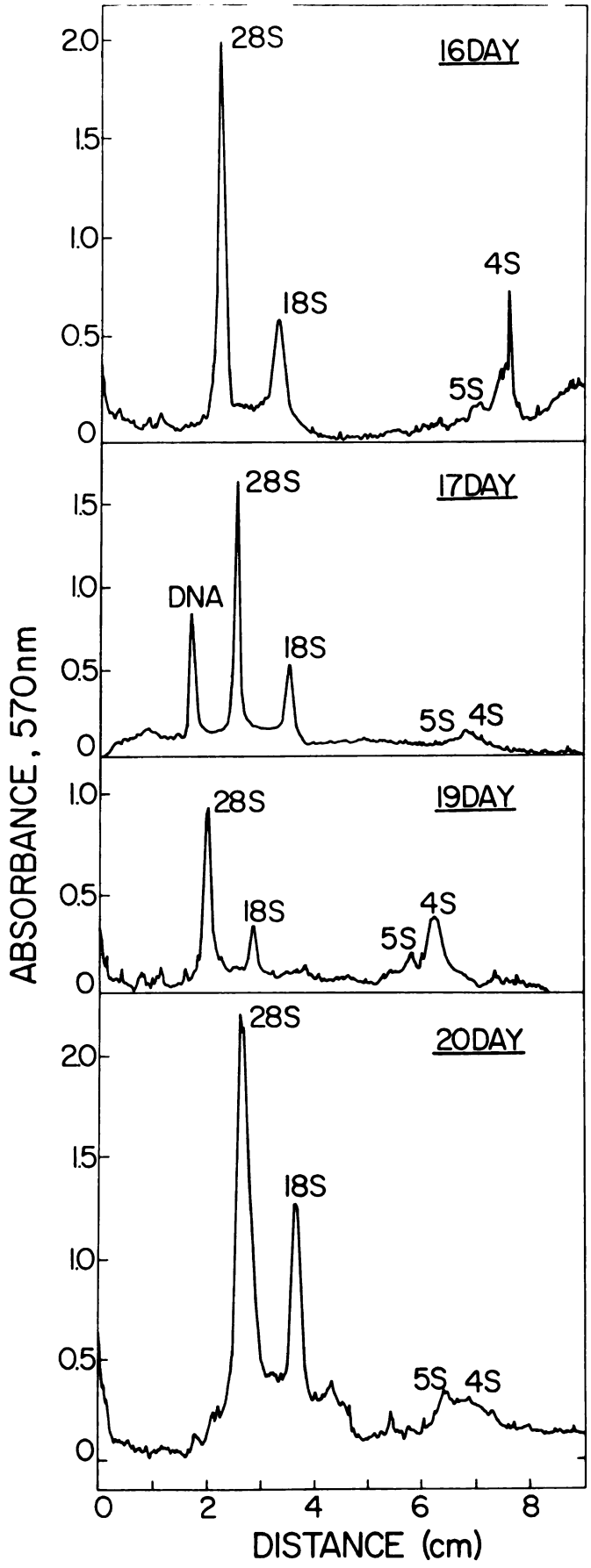

16DAY
28S
18S
4S
5S
17DAY
28S
DNA
18S
5S
4S
19DAY
28S
18S
4S
5S
20DAY
28S
18S
5S
4S
ABSORBANCE, 570nm
DISTANCE (cm)
0
2
4
6
8
0.5
1.0
1.5
2.0

Table 1. Relative amounts of major RNA species in RNA extracted from pancreatic and liver rudiments.[a]

Age	No. of determinations	28S (%)	18S (%)	5S (%)	4S (%)
Pancreas					
15	1	63	20	4	14
16	1	51	23	8	18
17	10	50.8 ± 8.5	26.2 ± 2.6	5.6 ± 2.2	17.4 ± 5.5
19	3	41 ± 2.9	21.5 ± 6.8	8.0 ± 1.6	29.5 ± 9.4
20	1	60	24	6.9	12
Liver					
15	1	53	24	7	16
17	3	49 ± 9.1	26.2 ± 2.0	6.5 ± 4.1	16.5 ± 6.5

[a]Areas of 28S, 18S, 5S and 4S RNA species in gel scans were estimated by approximating the area of a triangle inscribed in the peak. Values are given as percent of absorbance at 570 nm ± mean deviation.

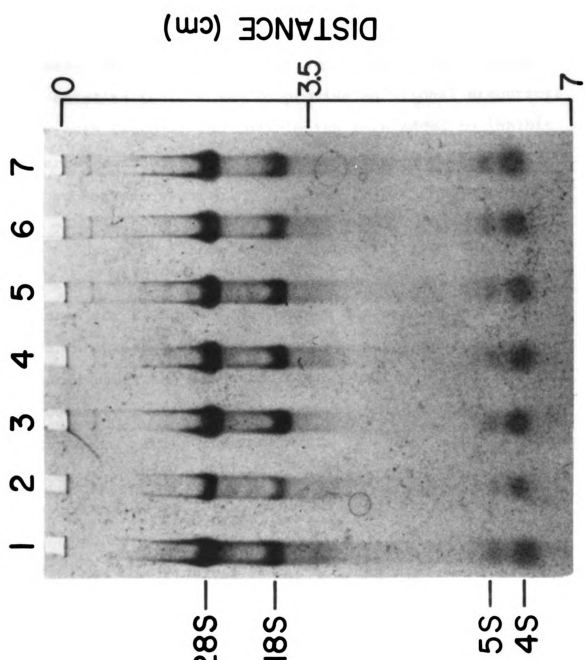
Organ Culture of Pancreatic Rudiments

Short Term Organ Culture

Seventeen-day pancreatic rudiments were aseptially removed and cultured intact in 1 ml of Eagle's MEM alone in a 12 X 75 mm sterile disposable centrifuge tube, for intervals up to 8 hours. Under these conditions, the tissue was completely submerged and the total rudiment mass varied from age to age. RNA degradation was used to evaluate tissue necrosis. Seventeen-day pancreases cultured for 15 minutes to 4 hours gave identical RNA patterns (Figure 5). Uptake of [^{3}H] uridine by 14, 17, and 19-day pancreas was linear for 4 hours with no indication of saturation kinetics. Electrophoretic analysis of phenol extractable counts revealed that none of the major RNA species were labelled by 4 hours. Most of the label was in fast migrating RNA with the remainder being dispersed throughout the gel. It was apparent that the labelling time was insufficient to permit labelling of rRNA. Using the same procedure, 17-day rudiments were cultured for 24 hours. At 12 hours in culture all the RNA extracted was completely degraded. This indicated that necrosis had occurred.

Four modifications were made in developing a satisfactory long term organ culture system; the rudiments were cut into pieces of approximately 5-15 µg of protein

Figure 5. Photograph of RNA from 17-day pancreases cultured for various intervals. RNA was extracted from 360 µg of tissue in 100 µl total volume as described in METHODS. Slot 1 contained RNA from 17-day liver; slot 2, RNA from 17-day pancreas cultured for 15 minutes; slot 3, RNA from 17-day pancreas cultured for 30 minutes; slot 4, RNA from 17-day pancreas cultured for 1 hour; slot 5, RNA from 17-day pancreas cultured for 2 hours; slot 6, RNA from 17-day pancreas cultured for 3 hours, and slot 7, RNA from 17-day pancreas cultured for 4 hours.


1
2
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4
5
6
7
0
3.5
7
DISTANCE (cm)
28S
18S
5S
4S

to allow labelled precursors and nutrients to penetrate the interior of the tissue; the tissue pieces were attached to a Millipore filter as a substrate and floated at the liquid gas interphase to facilitate oxygen-carbon dioxide exchange; fetal calf serum and supplemental amino acids were added to Eagle's MEM to provide additional precursors and growth factors; and antibiotics were added to inhibit bacterial contamination. These modifications are evaluated in a subsequent section.

Long Term Organ Culture

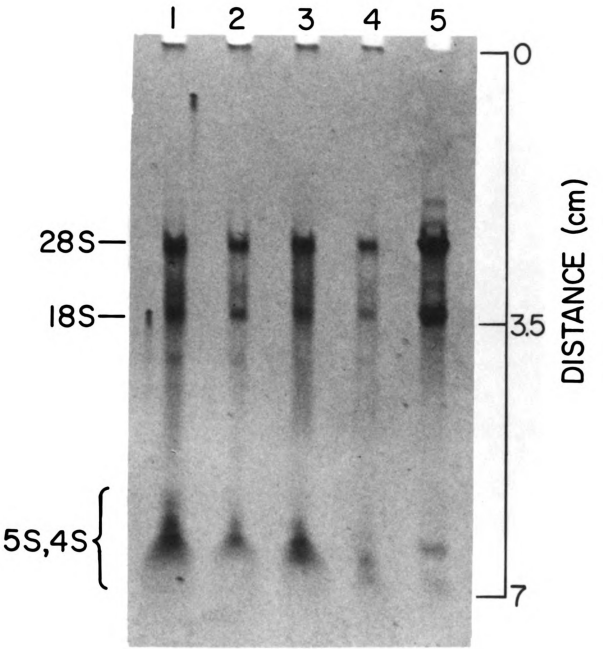
Using the above modifications distinct RNA bands were resolved from RNA extracted from 17-day pancreas cultured for 24 hours. To provide a control for assessing differentiation and tissue survival, adult pancreas was cultured under the same condition. Before this study, it was believed that adult rat pancreas could be successfully cultured only for short intervals. RNA from adult pancreas indicated no tissue necrosis. The migration patterns were comparable to 17-day pancreatic RNA (Figure 6).

RNA and Protein Synthesis in Fetal Rat Pancreas Cultured at pH 6.8

[^{3}H] Leucine Incorporation

When the air-CO_2 mixture was bubbled through water in the bottom of the 37°C incubator, it was observed that the equilibration pH of the tissue culture medium dropped

Figure 6. RNA from adult pancreas cultured for various periods at pH 6.8. RNA was extracted from approximately 400 µg of tissue under standard conditions. Slot 1 contained RNA from adult pancreas cultured for 4 hours; slot 2, 8 hours; slot 3, 12 hours; slot 4, 24 hours, and slot 5, 0.1 OD_{260} units of hepatoma rRNA.


1
2
3
4
5
0
28S
18S
3.5
DISTANCE (cm)
5S,4S
7

from 7.1 to 6.8. This unusual occurrence was probably due to a faulty gas meter which measured CO_2 incorrectly. This pH change upon equilibration was used to adjust the pH of the medium to 6.8. Labelling studies were conducted using 5 μCi/ml of [^{3}H] leucine in standard medium which contained 0.4 mM leucine. The added labelled leucine did not alter the total leucine concentration in the medium. Fourteen, 17, 18, 19, 20-day old pancreatic rudiments took up leucine into the soluble pool and incorporated it into TCA precipitable products at approximately the same rate (Figure 7). Leucine entered the soluble and protein pools at approximately the same rate at each age.

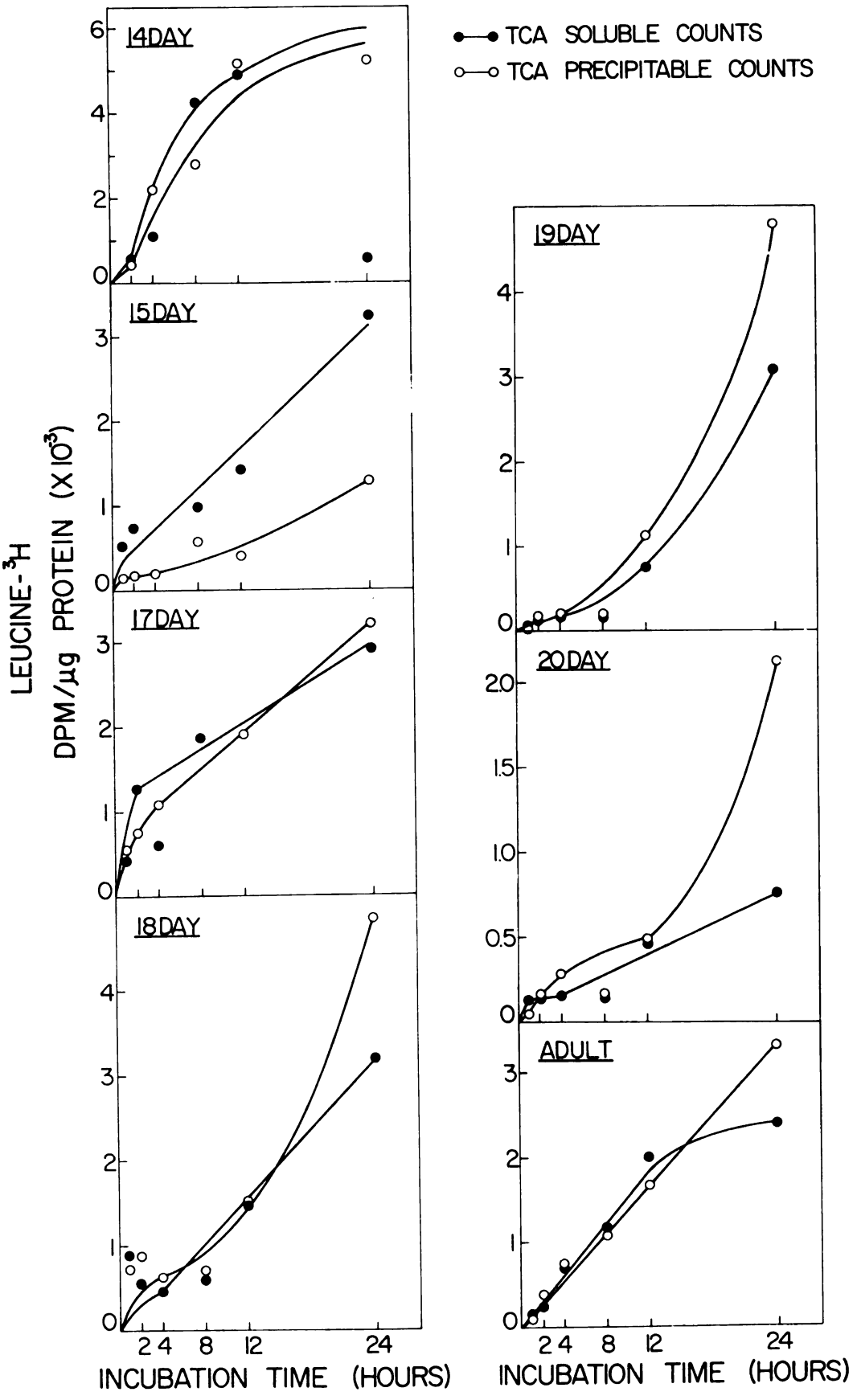
Fifteen-day pancreas incorporated leucine linearly for 24 hours with no indication of pool equilibration or steady state kinetics. At this age less than 30% of the label taken up by the tissue was incorporated into TCA precipitable material.

Adult pancreas incorporated leucine into the soluble pool for 12 hours and then the rate started to level off. Leucine was incorporated into protein at a linear rate for 24 hours and approximately the same rate at which it went into the soluble pool. This indicated that fetal and adult pancreas synthesized protein in culture.

[^{3}H] Uridine Incorporation

Uridine uptake analysis was conducted at 5 μci/ml of [^{3}H] uridine in complete medium. The Eagle's MEM contained no

Figure 7. Uptake of [^{3}H] leucine by fetal rat pancreas cultured at pH 6.8. Fetal rat pancreases of different embryonic ages were cultured at pH 6.8 on Millipore filters in 5 μCi/ml of [^{3}H] leucine as described in METHODS. (●—●) TCA soluble counts, (o—o) TCA precipitable counts.


TCA SOLUBLE COUNTS
TCA PRECIPITABLE COUNTS
14DAY
15DAY
17DAY
18DAY
19DAY
20DAY
ADULT
LEUCINE-³H
DPM/µg PROTEIN (X 10³)
INCUBATION TIME (HOURS)
INCUBATION TIME (HOURS)
2 4 8 12 24
2 4 8 12 24

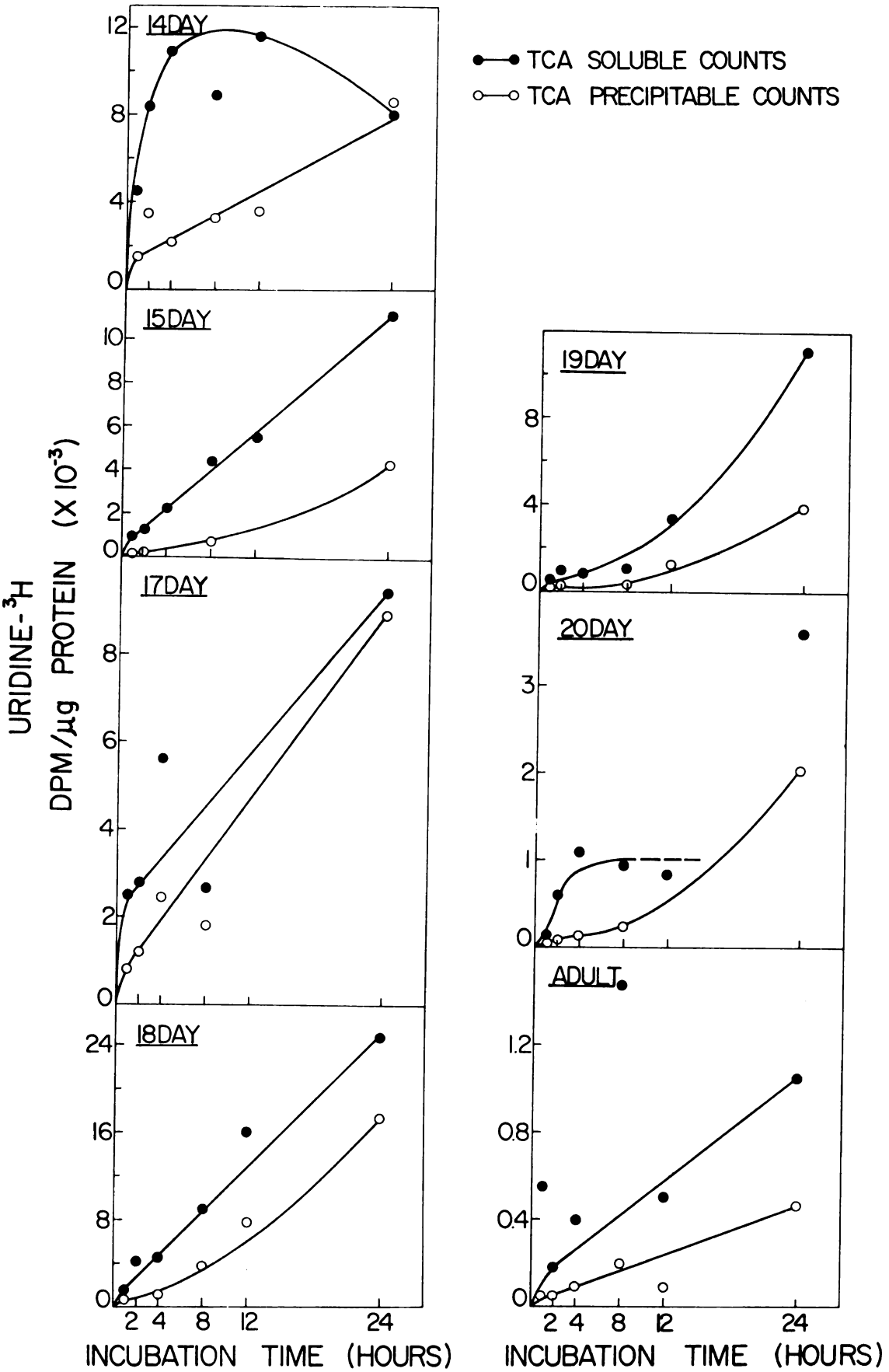
uridine or uracil other than labelled uridine and that in fetal calf serum. Fourteen-day pancreas incorporated uridine into the soluble pool at a linear rate for 4 hours (Figure 8). Incorporation into TCA precipitable material increased for 24 hours. During the first 8 hours 75% more uridine was incorporated into the soluble pool than in the insoluble material. By 24 hours the percentage of soluble counts was reduced to 50%. Seventy-five percent of the counts were soluble in 15 and 19-day pancreases cultured for 24 hours. At both ages, incorporation was linear for 24 hours. Seventeen and 18-day pancreases incorporated uridine linearly into both pools with 50% of the counts in the soluble pool. Adult and 20-day pancreases had a 24 hour linear uptake with a percentage in soluble counts of approximately 60%.

Alkaline resistant TCA percipitable counts were taken as a measure of [^{3}H] label incorporated into DNA. In uridine studies at pH 7.1 and 6.8, the percentage of alkaline resistant counts did not exceed 10% in rudiments labelled for 24 hours. This indicated that very little ^{3}H was incorporated into protein and DNA during the study.

Electrophoretic Separation of Labelled RNA from Fetal Pancreas

RNA extracted from 18-day pancreas labelled with [^{3}H] for 4 and 24 hours displayed a very low number of

Figure 8. Uptake of [^{3}H] uridine by fetal rat pancreas cultured at pH 6.8. Fetal rat pancreases of different embryonic ages were cultured at pH 6.8 on Millipore filters in 5 μCi/ml of [^{3}H] uridine as described in METHODS.


14DAY
15DAY
17DAY
18DAY
19DAY
20DAY
ADULT
TCA SOLUBLE COUNTS
TCA PRECIPITABLE COUNTS
URIDINE-^{3}H
DPM/µg PROTEIN (X 10^{-3})
INCUBATION TIME (HOURS)
INCUBATION TIME (HOURS)

counts (Figure 9). After 4 hours of labelling, none of the major RNA species were distinctly labelled. The [^{3}H] uridine appeared as a broad peak in the region of 28S and 18S RNA and as a double peak in the 4S region. After incubating 24 hours in the presence of [^{3}H] uridine, 28S and 18S labelled peaks could be distinguished. A major low molecular peak migrated in the region of 5S RNA.

Adult pancreatic RNA labelled for 8 hours gave two main peaks after electrophoresis (Figure 10). One migrated with a molecular weight greater than 28S and the other migrated around 10S. In 12 hours the high molecular weight peak migrated closer to 28S, a small percentage of label appeared in a 28S peak and a double peak migrate near 5S RNA. The amount of [^{3}H] uridine incorporated into rRNA was low.

Comparable results were obtained with 17 and 20-day pancreases. None of the rRNA peaks were labelled until 12 hours of incubation, and a number of unusual peaks were detected. The results suggested that at pH 6.8 rRNA synthesis in fetal and adult pancreases is diminished. Furthermore, much of the radioactivity was incorporated into RNA species which normally constitute a small percent of total RNA.

Figure 9. Profiles of labelled RNA from 18-day pancreatic rudiments maintained in culture at pH 6.8 for 4 and 24 hours. Approximately 50-100 µg of tissue was incubated with 5 µCi/ml of [^{3}H] uridine in complete medium containing Eagle's MEM; 10% fetal calf serum, antibiotics and supplemental animo acids at pH 6.8 as described in METHODS. After incubation at 37°C for 4 and 24 hours, RNA was extracted and subjected to electrophoresis and the gels were stained and scanned. Distribution of radioactivity was determined as described in METHODS.

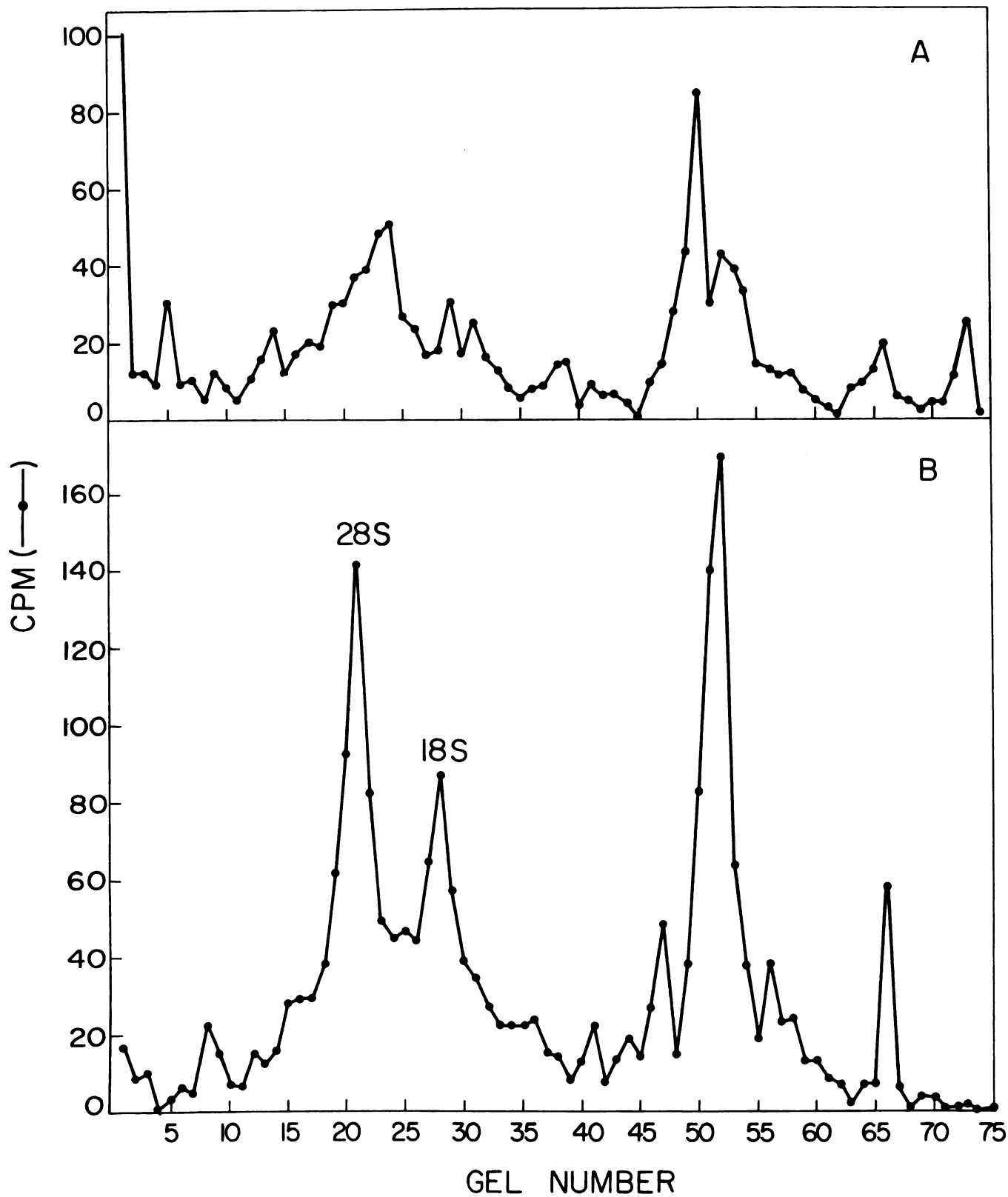

A
B
28S
18S
CPM (—●—)
GEL NUMBER
0
20
40
60
80
100
120
140
160
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75

Figure 10. Profiles of labelled RNA from adult pancreas maintained at pH 6.8 for 8 and 12 hours. Adult pancreas was labelled and the RNA extracted and subjected to electrophoresis as described in METHODS.(●—●) 8 hours, (o—o) 12 hours, (-----) absorbance.

cpm
250
200
150
100
50
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
SLICE NUMBER
0.5
1.0
ABSORBANCE, 570 nm (----)

Protein and RNA Synthesis by Fetal Rat Pancreas Cultured at pH 7.1

Rates of Protein and RNA Synthesis in 19-Day Pancreas Cultured Under Different Culture Conditions

By adjusting the air-CO_2 mixture flow, the pH was stabilized at pH 7.1. A series of experiments was carried out to determine the effects of culture conditions on apparent rates of [^{3}H] uridine incorporation into RNA and [^{3}H] leucine incorporation into protein.

Nineteen-day pancreatic rudiments were cultured for 24 hours in medium without supplemental amino acids and compared with rudiments cultured with complete MEM. L-glutamine in liquid medium is unstable and tend to break down upon storage. To alleviate this problem, this amino acid was added in the supplemental amino acids. Some investigators believe that L-arginine plays a paramount role in the survival of tissue in culture (70). The special function of the amino acid is undefined. Some believe it is involved in histone control of DNA transcription; others believe it is no more important than other amino acids. It was assumed that arginine was essential and it was added in the supplemental amino acids. Because Parsa et al. demonstrated a requirement for methionine in cultured fetal pancreas, methionine was included in the supplemental amino acids.

Electrophoretic analysis of 19-day pancreatic RNA labelled without supplemental amino acids gave profiles comparable to 19-day pancreas cultured with supplemental amino

Figure 11. Profiles of labelled RNA from 19-day fetal pancreas maintained in culture at pH 7.1 for 4 (A) and 12 (B) hours in the absence of supplemental amino acids. Nineteen-day pancreas (150-200 µg) was incubated on Millipore filters in Eagle's MEM-10% fetal calf serum with and without supplemental amino acids: 0.12 mM L-arginine, 0.2 mM l-methionine and 2 mM L-glutamine. After incubating at 37°C for 4 and 12 hours in 5 µCi/ml of [^{3}H] uridine, the tissues were rinsed and the labelled RNA was extracted and resolved by electrophoresis as described in Figure 4. The gel was stained and scanned as described in METHODS. The distribution of radioactivity was then determined. (----) RNA from rudiments labelled in medium without supplemental amino acids. (●—●) RNA from rudiments labelled in medium with supplemental amino acids.

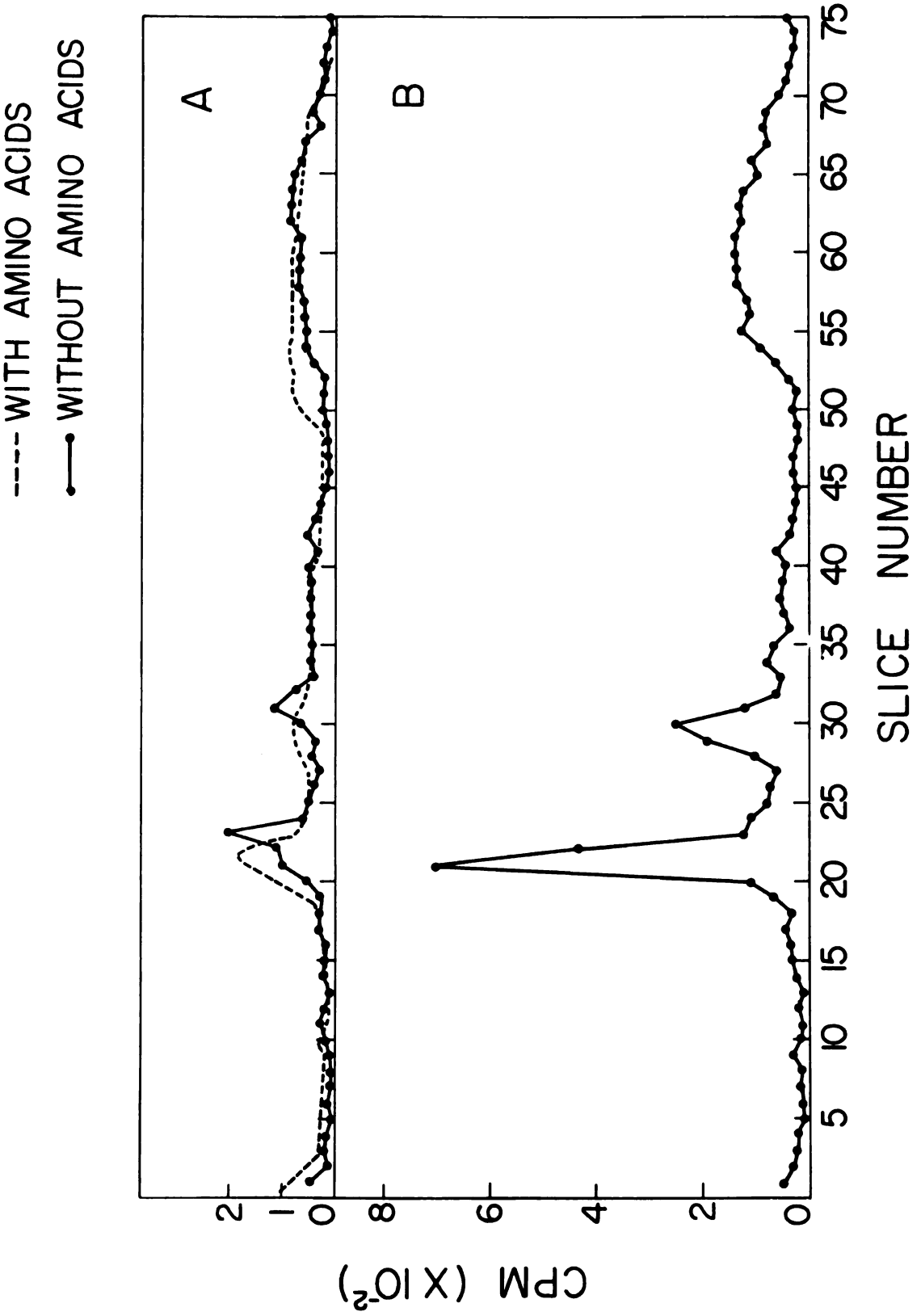
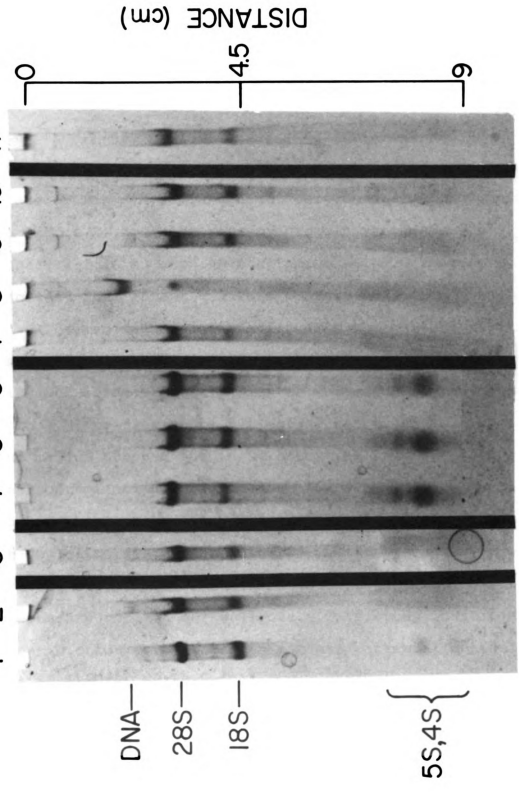

---WITH AMINO ACIDS
—•—WITHOUT AMINO ACIDS
A
B
CPM (X 10⁻²)
SLICE NUMBER

Figure 12. Photography of RNA from 19-day pancreases cultured at pH 7.1 for various intervals with and without supplemental amino acids. Tissues (150-200 g) were incubated and RNA was extracted in 100 µl total volume as described in METHODS. Slot 1 contained 0.1 OD_{260} units of hepatoma rRNA. Slots 2 through 6 contained RNA from 19-day pancreas cultured without additional amino acids in a standard concentration of MEM and fetal calf serum: slot 2, cultured for 4 hours; slot 3, cultured for 12 hours; slot 4, cultured for 15 minutes; slot 5, cultured for 30 minutes, and slot 6, cultured for 1 hour. Slots having RNA from 19-day pancreas cultured with supplemental amino acids (standard culture conditions) were: slot 7, cultured for 4 hours; slot 8, cultured for 8 hours; slot 9, cultured for 12 hours; slot 10, cultured for 24 hours, and slot 11, cultured for 36 hours.



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Tissue
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9-day
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hours.

acids (Figure 11). The RNA patterns suggest no differences in the migration of the RNA extracted from tissue cultured under the two conditions (Figure 12). The pattern shown in slot 8 depicts degradation. This could have occurred during tissue necrosis or RNA extraction. Because the RNA extracted from tissue cultured for longer periods was undegraded, the degradation probably occurred during extraction. The rate of RNA synthesis did not vary under the two conditions (Table 2), but the rate of protein synthesis was increased 2-fold in supplemental amino acids. Therefore, we included them in our standard culture medium.

The removal of antibiotics from the culture medium had no effect on the initial rate of RNA and protein synthesis, but after 4 hours a drastic reduction in the uptake of both [^{3}H] uridine and [^{3}H] leucine was observed. The pH of the medium dropped and in 24 hours the trubidity of bacterial contamination was evident. The drop in label uptake could have been caused by cellular necrosis due to the acid pH or depletion of the label in the medium by rapidly proliferating bacteria. It was thought that antibiotics were not necessary for 24 hour cultures. This study indicated that antibiotics were needed to prevent bacterial contamination of growing cultures.

Chick embryo extract is often added to culture media to provide additional growth factors. The extract is usually the supernatant fraction from a sedimented

Table 2. Rates of [^{3}H] uridine and [^{3}H] leucine incorporation into TCA precipitable material in cultured pancreatic tissues.[a]

Change in Culture Conditioning	[^{3}H] Leucine[b]		[^{3}H] Uridine[b]	
	DPM/cell/hr(10^3)	DPM/μg Protein/hr.	DPM/cell/hr(10^3)	DPM/μg Protein/hr.
19-day pancreas				
Standard medium	114.8 ± 15.2[a]	310 ± 40	87.0 ± 5.5	235 ± 15
(duplicate culture)	116.6 ± 13.0	315 ± 35		
24 hour preincubation (standard medium)	83.0 ± 27.0	166 ± 54	70. ± 35	140 ± 70
No supplemental amino acids	42.5 ± 9.3	115 ± 25	83.3 ± 20.4	225 ± 55
No antibiotics	101.8 ± 35.7	275 ± 95	96.2 ± 22.3	260 ± 60
+3% embryo extract	118.5 ± 18.5	320 ± 50	79.6 ± 9.2	215 ± 25
Standard medium (pH 6.8)	40.0 ± 11.8	108 ± 32	42.9 ± 14.5	203 ± 52
No serum (pH 6.8)		78 ± 32		58 ± 7
Adult (pH 6.8)	96.8 ± 9.4	155 ± 15	126.8 ± 32.5	203 ± 52
Adult (pH 7.1)	200. ± 43.7	155 ± 15	126.8 ± 32.5	116 ± 39

[a]Tissues were cultured under standard conditions unless otherwise indicated.

[b]± = mean deviation of the average of the minimum and maximum slope.

homogenate of chicken embryos and may contain a variety of factors ranging from growth promoters to essential vitamins. Addition of 3% embryo extract had no effect on the rate of RNA and protein synthesis (Table 3). This suggested that either the necessary growth factors for pancreas cultured for 24 hours are provided in the other components of the medium, or that they are not needed by the tissue at the ages studied. Cultures incubated more than 2 days may require embryo extract. Nineteen-day pancreases were cultured for 24 hours in the absence of [^{3}H] uridine and [^{3}H] leucine and then transferred to labelled medium for 24 hours. The rates obtained from the uptake curves were similar to control values (Table 2). This showed that the studies conducted in 24 hours were not performed at the limit of *in vitro* survival of the tissue.

Fetal calf serum was added to tissue culture medium to provide physiological hormones and factors. Addition of 10% fetal calf serum to cultures resulted in a 0.3-fold increase in protein synthesis and a 3-fold increase in RNA synthesis (Table 2). Fetal calf serum is heterogenous in composition, and therefore, the stimulation could have been caused by one or more factors.

[^{3}H] Leucine Incorporation

Kinetic studies of [^{3}H] leucine incorporation described in the preceding section were repeated. Equilibration of the

soluble pools was generally apparent within 4 to 12 hours (Figure 13). This is in contrast with the results obtained at suboptimal pH. The incorporation of counts into TCA insoluble material increased linearly for approximately 24 hours. The soluble pool accounted for 30-50% of the total counts in the pancreatic homogenates after 12 hours. The uncertainty in the estimates of radioactivity in the soluble pool, obtained as the difference of two relatively large numbers, is rather high. This may account for the scatter in these points as observed, for example, in the 19-day tissues.

The apparent rate of [^{3}H] leucine incorporation into TCA precipitable material varies throughout differentiation of the pancreas as shown by Figure 14. At pH 6.8, the apparent rate of incorporation (dpm/µg protein/hr) decreased between 14 and 15 days of gestation. The rate increased several fold to a maximum of 200 dpm/µg protein/hr at 17 days of gestation, then declined to the 15-day level after 20 days of gestation. The rate of protein synthesis in the adult pancreas was comparable to 18 and 19-day rates.

At pH 7.1, the apparent rate increased from approximately 150 dpm/µg protein/hr at 15 and 16 days of gestation to a maximum of 450 dpm/µg protein/hr then declined to the 15-day level. The rates were approximately 3-fold greater than the pH 6.8 values at each age. Seventeen-day

Figure 13. Uptake of [^{3}H] leucine by fetal rat pancreases cultured at pH 7.1. Fetal rat pancreases of different embryonic ages were cultured at pH 7.1 on Millipore filters in 5 μCi/ml of [^{3}H] leucine as described in METHODS. (●—●) TCA soluble counts, (o—o) TCA precipitable counts.

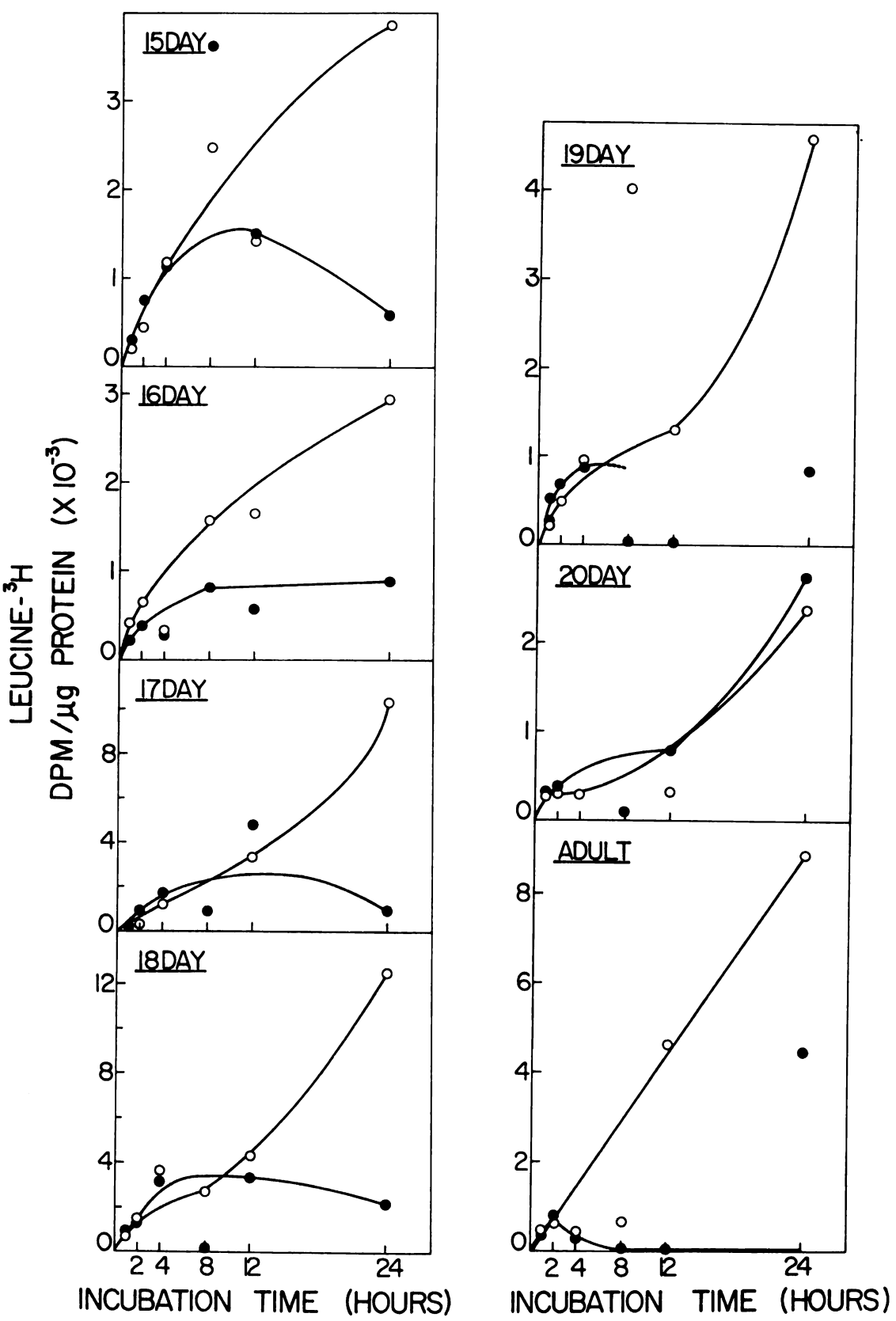
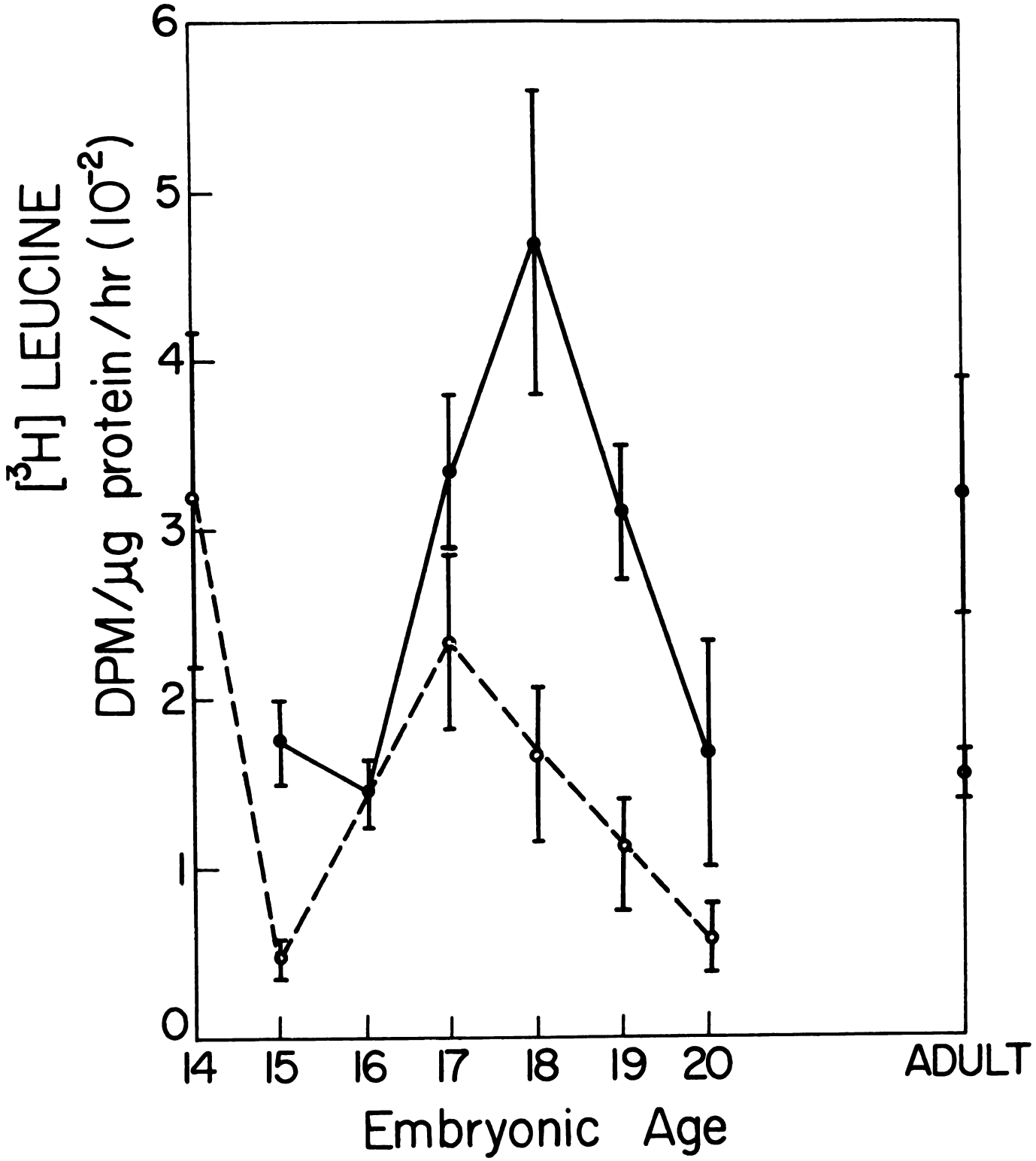

TCA SOLUBLE COUNTS
TCA PRECIPITABLE COUNTS
15DAY
16DAY
17DAY
18DAY
19DAY
20DAY
ADULT
LEUCINE-^{3}H
DPM/µg PROTEIN (X 10^{-3})
INCUBATION TIME (HOURS)
INCUBATION TIME (HOURS)

Figure 14. Apparent rates of [^{3}H] leucine incorporation per microgram of protein. The average of the maximum and minimum slopes were taken for each incorporation curve for TCA precipitable counts at pH 6.8 (Figure 6) and pH 7.1 (Figure 11), and plotted as a function of embryonic age. (●——●) pH 7.1, (o---o) pH 6.8. The bars indicate the maximum and minimum slope.


[3H] LEUCINE
DPM/µg protein/hr (10-2)
0
1
2
3
4
5
6
14
15
16
17
18
19
20
ADULT
Embryonic Age

rudiments and adult pancreatic tissue were least affected by the change in pH.

As the pancreas differentiates, the ratio of protein to DNA content in the tissue increases. The cells become larger and more specialized, as indicated by the formation of new membrane structures and the accumulation of tissue specific products such as amylase. The rates of incorporation per microgram of protein were converted to rates per cell by using the quantity of protein per cell calculated for each embryonic age by Rutter et al. (unpublished data). When the apparent rates of protein synthesis per cell are plotted as a function of embryonic age, there is an apparent transition between 16 and 19 days of gestation at both pH 7.1 and 6.8 (Figure 15). At pH 7.1 as well as 6.8, 19-day old tissues incorporated 8-fold more [^{3}H] leucine into protein per cell than did 15 or 16-day tissue. The greatest rate of protein synthesis per cell occurred in adult pancreases which was 2-fold greater than the 19-day rate at pH 7.1.

[^{3}H] Uridine Incorporation

Fifteen-day pancreatic rudiments incorporated [^{3}H] uridine into TCA insoluble material at a linear rate for 12 to 24 hours during the developmental transition (Figure 16). The amount of label in the TCA soluble pool increased at a linear rate up to 24 hours for several sets of tissue, e.g., rudiments from 15 and 17-day old embryos. In other

Figure 15. Apparent rates of [^{3}H] leucine incorporation per cell. The apparent rates in Figure 14 were converted to rates per cell by using the protein per cell values calculated by Rutter, et al. (unpublished data). (●—●) pH 7.1, (o---o) pH 6.8. The bars indicate the maximum and minimum slopes.

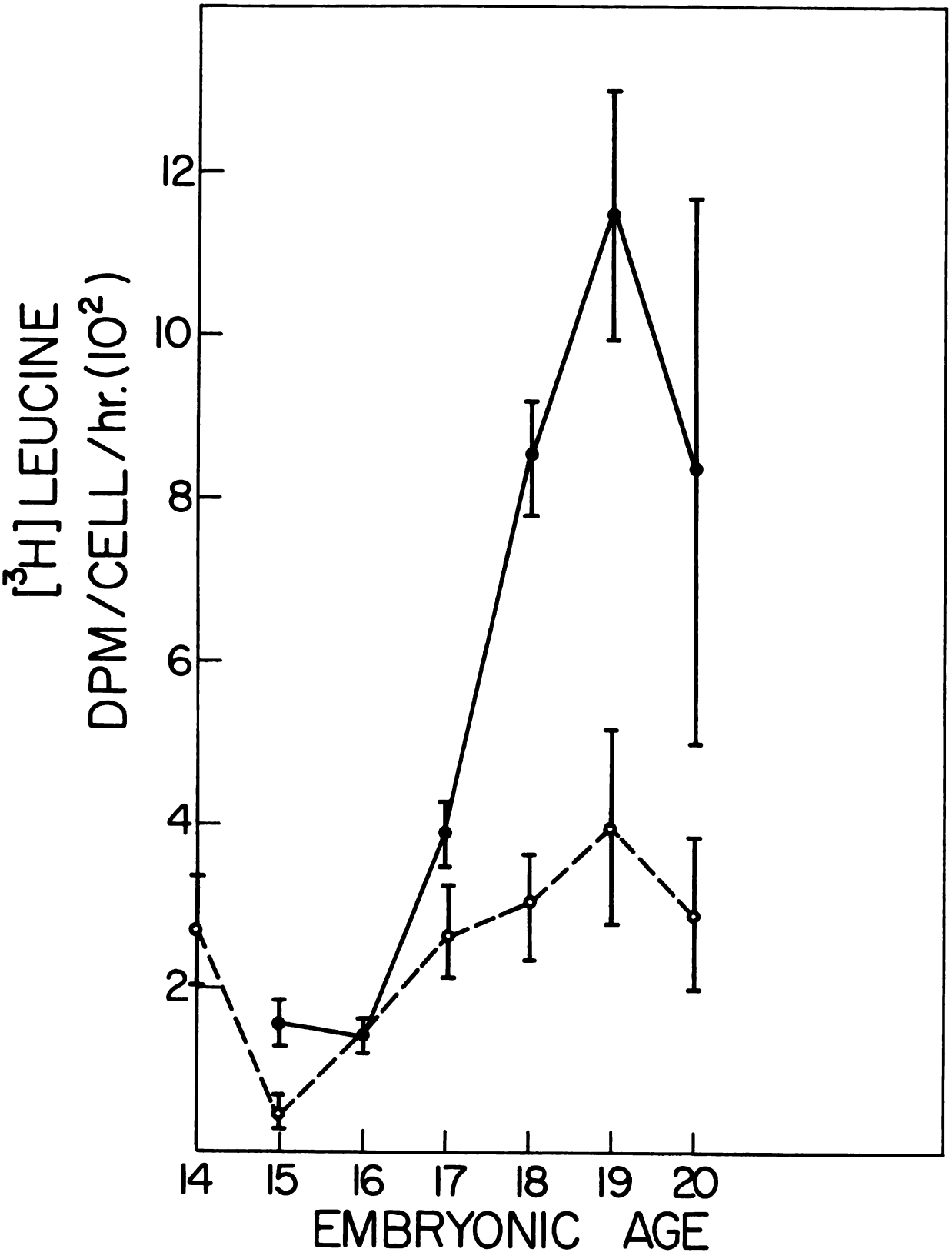
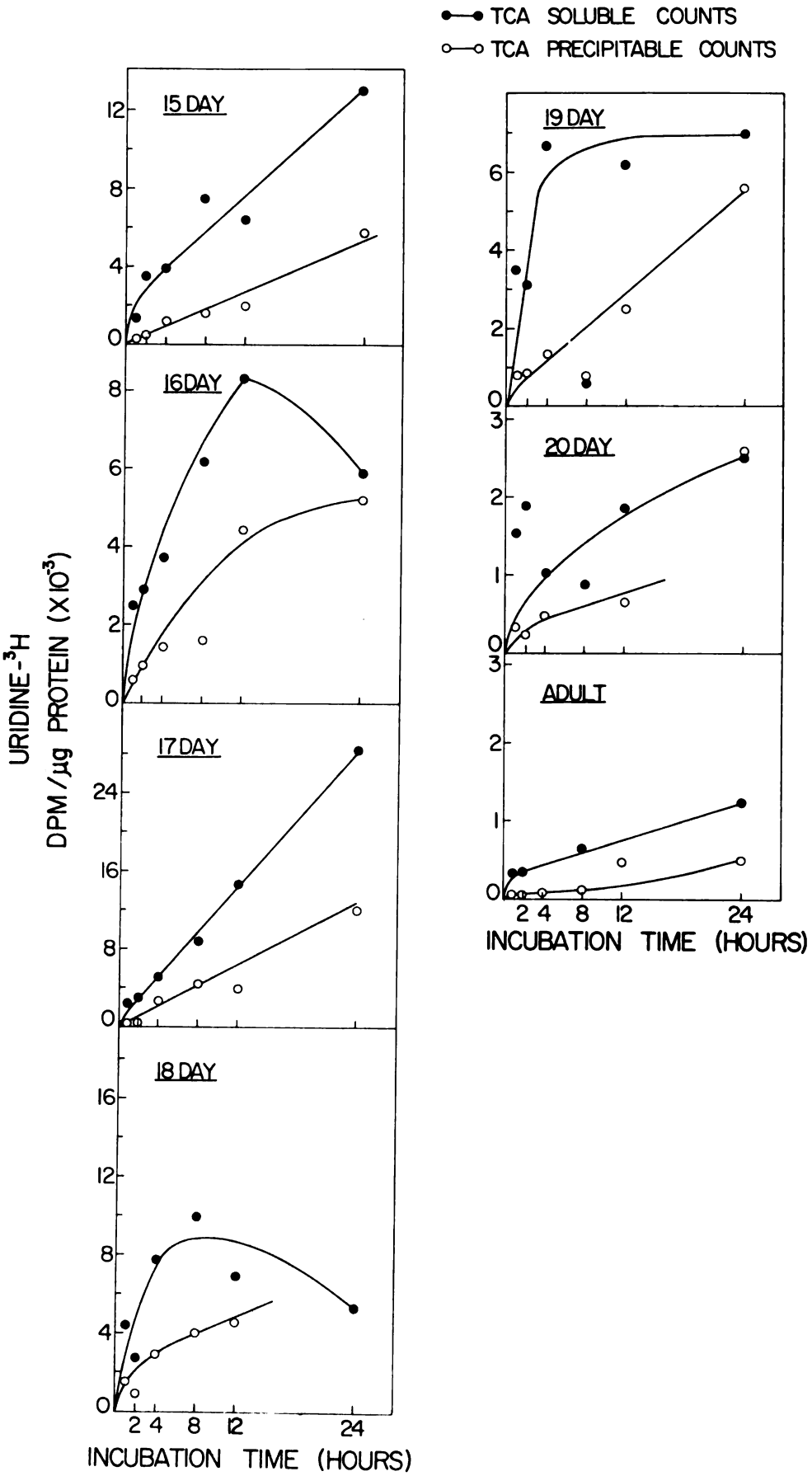

[3H] LEUCINE
DPM/CELL/hr.(10²)
12
10
8
6
4
2
14
15
16
17
18
19
20
EMBRYONIC AGE

Figure 16. Uptake of [^{3}H] uridine by fetal rat pancreases cultured at pH 7.1. Fetal rat pancreases of different embryonic ages were cultured on Millipore filters in 5 μCi/ml of [^{3}H] uridine as described in METHODS.


TCA SOLUBLE COUNTS
TCA PRECIPITABLE COUNTS
15 DAY
16 DAY
17 DAY
18 DAY
19 DAY
20 DAY
ADULT
URIDINE-^{3}H
DPM/µg PROTEIN (X10^{-3})
INCUBATION TIME (HOURS)
2 4 8 12 24

cases, the soluble pool apparently equilibrated in 4 to 12 hours, e.g., 18 and 19-day old rudiments. After 12 hours incubation, approximately 30% of the [^{3}H] uridine in the homogenate was TCA precipitable.

The apparent rates of [^{3}H] uridine incorporation (dpm/mg protein/hr) were plotted as a function of age and the data are summarized in Figure 17. Changing the pH from 6.8 to 7.1 caused only a slight increase in the apparent rates of RNA synthesis, although the apparent rates of protein synthesis were extensively affected (Figure 13). Again, in contrast to protein synthesis, the maximal rate in RNA synthesis occurred on day 18, one day before the transition in protein synthesis. From day 15 to day 18, the rate of RNA synthesis increased 5-fold at pH 6.8 and 2.5-fold at pH 7.1.

Calculating the rates of [^{3}H] uridine incorporation per cell instead of per microgram of protein did not alter the rate curve; maximal rates were attained after day 18 of gestation (Figure 18). Relative to DNA content, the maximal apparent rate of RNA synthesis occurred a day before the maximal rate in protein synthesis; relative to protein content, the apparent maximal rate of synthesis occurred on the same day. These data, therefore, suggest that a transition in both the apparent rate of RNA synthesis and the apparent rate of protein synthesis occurs in the embryonic rat pancreas midway through cytodifferentiation.

Figure 17. Apparent rates of [^{3}H] uridine incorporation per microgram of protein. The average of a maximum and minimum slope was taken for each incorporation curve for TCA precipitable counts at pH 6.8 (Figure 7) and pH 7.1 (Figure 14), and plotted as a function of embryonic age. (●——●) pH 7.1, (o---o) pH 6.8. The bars indicate the maximum and minimum slopes.

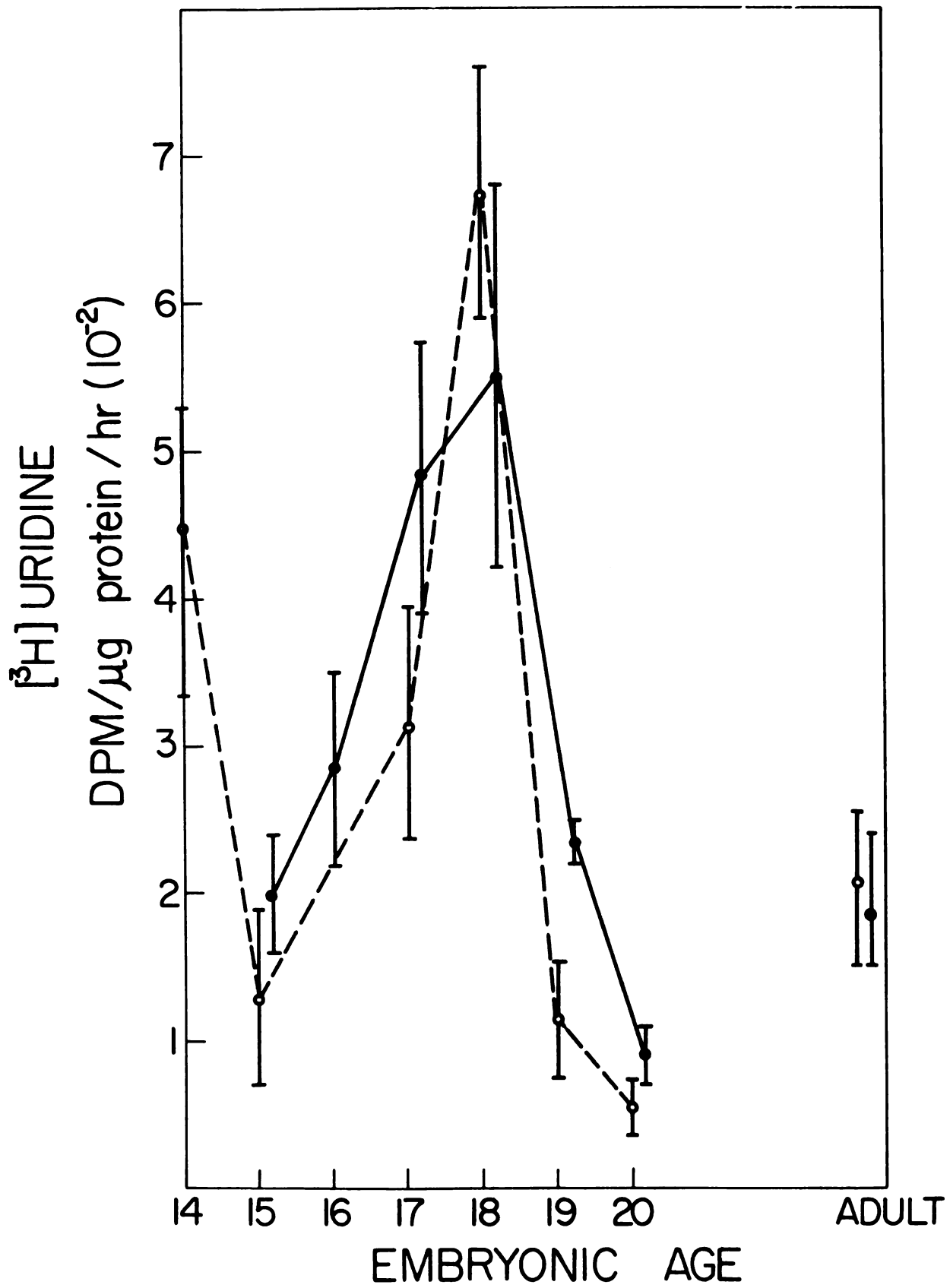
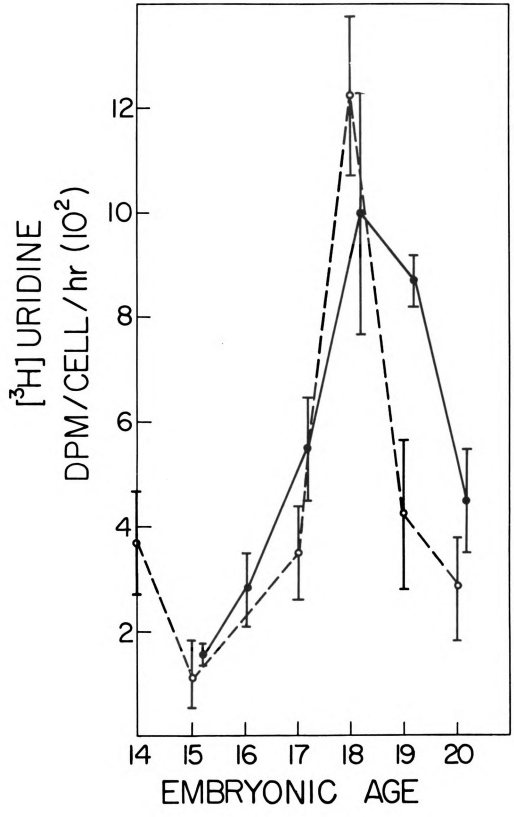

[3H] URIDINE
DPM/µg protein / hr (10-2)
7
6
5
4
3
2
1
14
15
16
17
18
19
20
ADULT
EMBRYONIC AGE

Figure 18. Apparent rates of [^{3}H] uridine incorporation per cell. The apparent rates in Figure 17 were converted to rates per cell by using the protein per cell values calculated by Rutter, et al. (unpublished data). (●——●) pH 7.1, (o——o) pH 6.8. The bars indicate the maximum and minimum slopes.


[3H] URIDINE
DPM/CELL/hr (10²)
12
10
8
6
4
2
14
15
16
17
18
19
20
EMBRYONIC AGE

An increased rate of synthesis per cell precede an increased rate of protein synthesis per cell.

Electrophoretic Analysis of Pancreatic RNA from Fetal Rat Pancreas Labelled In vitro at pH 7.1

Analysis of RNA from 14-Day Old Pancreatic Rudiments

RNA was extracted from 14-day labelled with [^{3}H] uridine in vitro at pH 7.1 and electrophoretically analyzed on an agarase-acrylamide gel. This experiment is summarized in Figure 19. The photograph of the gel indicates that each of the major rRNA species, as well as 4S RNA, are distinct bands. There is little evidence for degradation, even after 24 hours in culture.

After the gel was photographed, it was cut into 5 mm strips, each of which corresponded with a sample slot. Representative electropherograms were scanned to determine the position of 28S and 18S rRNA as described in METHODS. The strips were cut into sections of approximately 1 mm and the distribution of radioactivity was determined. These data are summarized by Figure 20. Within 4 hours of culture in labelled medium, peaks corresponding to 28S and 18S RNA could be readily distinguished. After 8 hours of continuous labelling there was little variation in the distribution of radioactivity. Throughout the labelling period, a large number of counts migrated with an apparent

Figure 19. Photograph of RNA from 14-day pancreases cultured for various intervals at pH 7.1 in 5 μCi/ml of [^{3}H] uridine. RHA was extracted from 150-200 μg of tissue as described in METHODS. Slot 1 contained RNA from adult liver (marker); slot 2, RNA from 14-day pancreas cultured for 24 hours; slot 3, 14-day pancreas cultured for 12 hours; slot 4, RNA from 14-day pancreas cultured for 8 hours with adult liver RNA as carrier; slot 5, RNA from 14-day pancreas cultured for 4 hours and adult liver RNA.

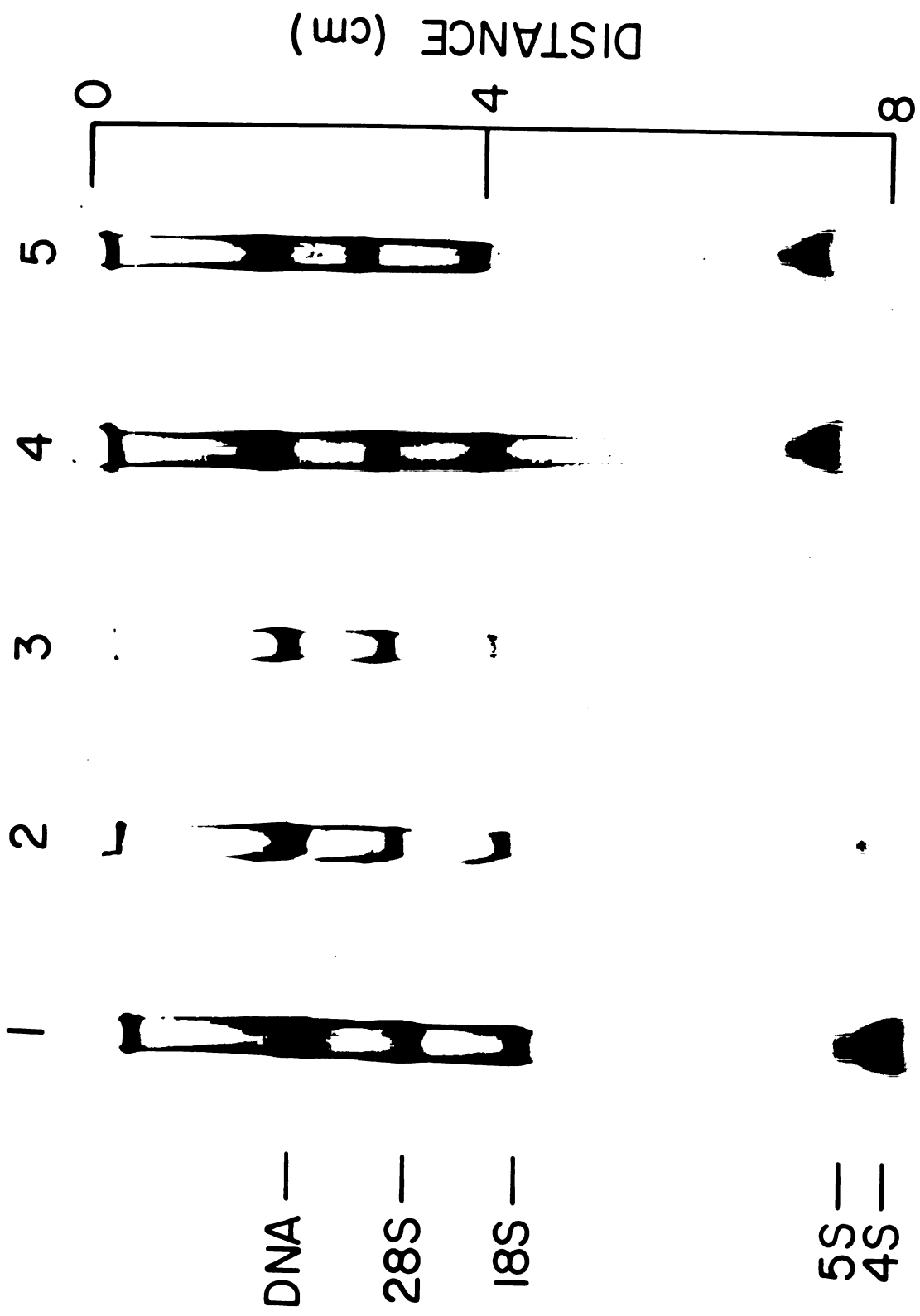
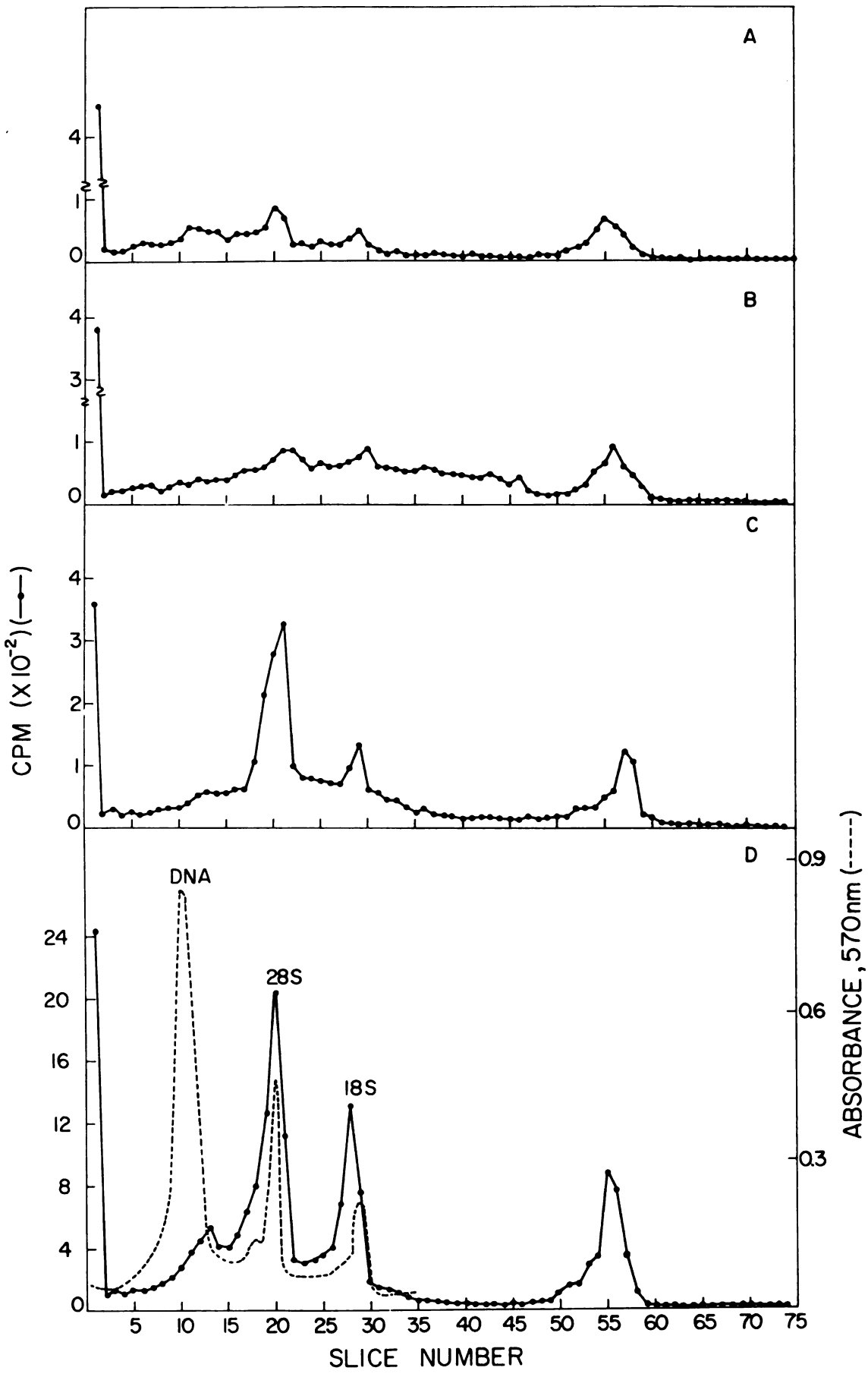

DISTANCE (cm)
0
4
8
5
4
3
2
1
DNA
28S
18S
5S
4S

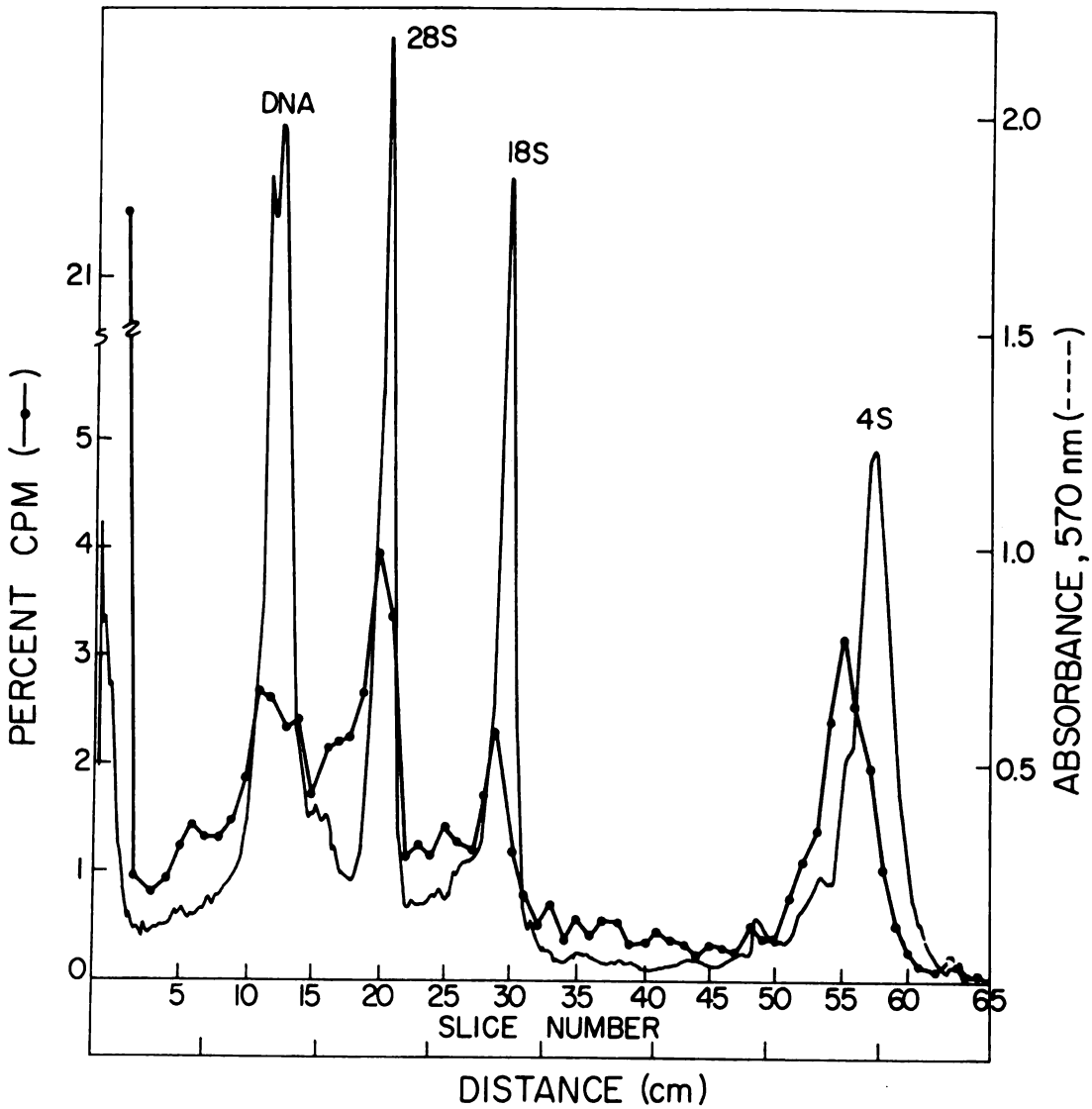
Figure 20. Distribution of radioactivity in RNA from 14-day fetal pancreas maintained at pH 7.1. The gels shown in Figure 19 were cut into 5 mm wide strips, cut into 1 mm slices and counted for radioactivity. Representative gels were scanned to indicate position of 28S and 18S RNA. Labelling times were: A, 4 hours; B, 8 hours; C, 12 hours, and D, 24 hours. (●——●) counts per minute, (----) absorbance.


A
B
C
D
DNA
28S
18S
CPM (X10⁻²)(—)
ABSORBANCE, 570nm (-----)
SLICE NUMBER

molecular weight greater than 28S. Because the division time for mammalian cells in culture is 12 to 24 hours, it is unlikely that the counts observed in this high molecular weight region of the gel at 4 and 8 hours are in DNA. This suggested that the counts migrating with an apparent molecular weight greater than 28S at 4 and 8 hours are probably in RNA, at the later times, they are probably in DNA. During the extraction of labelled RNA, some of the denatured protein at the interphase of the phenolic and aqueous phases is removed with the small volume of aqueous phase. This insoluble contaminate contains trapped [^{3}H] uridine and represents the high number of counts at the origin of the gels.

Green, et al. (71) noted that the amount of labelled RNA varied by more than 2-fold for a given set of mammary gland explants undergoing hormone induced differentiation. To compare radioactive profiles of labelled RNA for different sets of tissue, they expressed their results as percent of total counts per minute. To provide a comparable analysis, the data from Figure 20 was treated similarly and are summarized in Figure 21. Figure 22 compares the percent of counts per minute to the absorbance of stained gels. A relatively large percentage of count migrates with a molecular weight greater than 28S. As mentioned earlier, the counts are assumed to be in RNA because of the early labelling. However, the percentage

Figure 21. Percent distribution of radioactivity in 14-day pancreases labelled with [^{3}H] uridine for 4 hours. The data from the 4 hour profile of Figure 20 were converted to percentages and plotted with an absorbance scan from that stained gel.


28S
DNA
18S
4S
21
5
4
3
2
1
0
2.0
1.5
1.0
0.5
5
10
15
20
25
30
35
40
45
50
55
60
65
PERCENT CPM (—•—)
ABSORBANCE, 570 nm (----)
SLICE NUMBER
DISTANCE (cm)

of counts in this region and latter time is probably in DNA. From 4 hours to 24 hours it is expected that this percentage becomes less RNA and more DNA. So, at an interior period it is difficult to assess the contribution of DNA and RNA to the components migrating behind 28S RNA. Because of mechanical problems involving the gel slicer, the peak in the radioactive profile did not line up with the peaks on the absorbance scan. Alignment of the 28S and 18S peaks resulted in a misalignment of the 4S peak. After 4 hours of labelling, a large percentage of counts migrates in the 5S and 4S region of the gel with a relatively small percentage in the 28S and 18S peaks. This suggests that 4S and 5S RNA is labelled faster than 18S and 28S RNA.

Figure 22 shows a percent comparison of the radioactivity distribution throughout the 24 hour labelling period. The large percentage of high molecular weight counts are more evident. The percentage of counts in major peaks are summarized in Table 3. After 24 hours the percentage of counts in 28S and 18S RNA increased 2-fold. The labelling ratios resemble the absorbance profile of total RNA described earlier.

Analysis of RNA from 17-Day Pancreatic Rudiments

Seventeen-day old pancreatic rudiments were cultured with [^{3}H] uridine for periods up to 24 hours as

Figure 22. Percent distribution of radioactivity in 14-day pancreases labelled with [^{3}H] uridine. The data in Figure 20 were converted to percent of total counts.

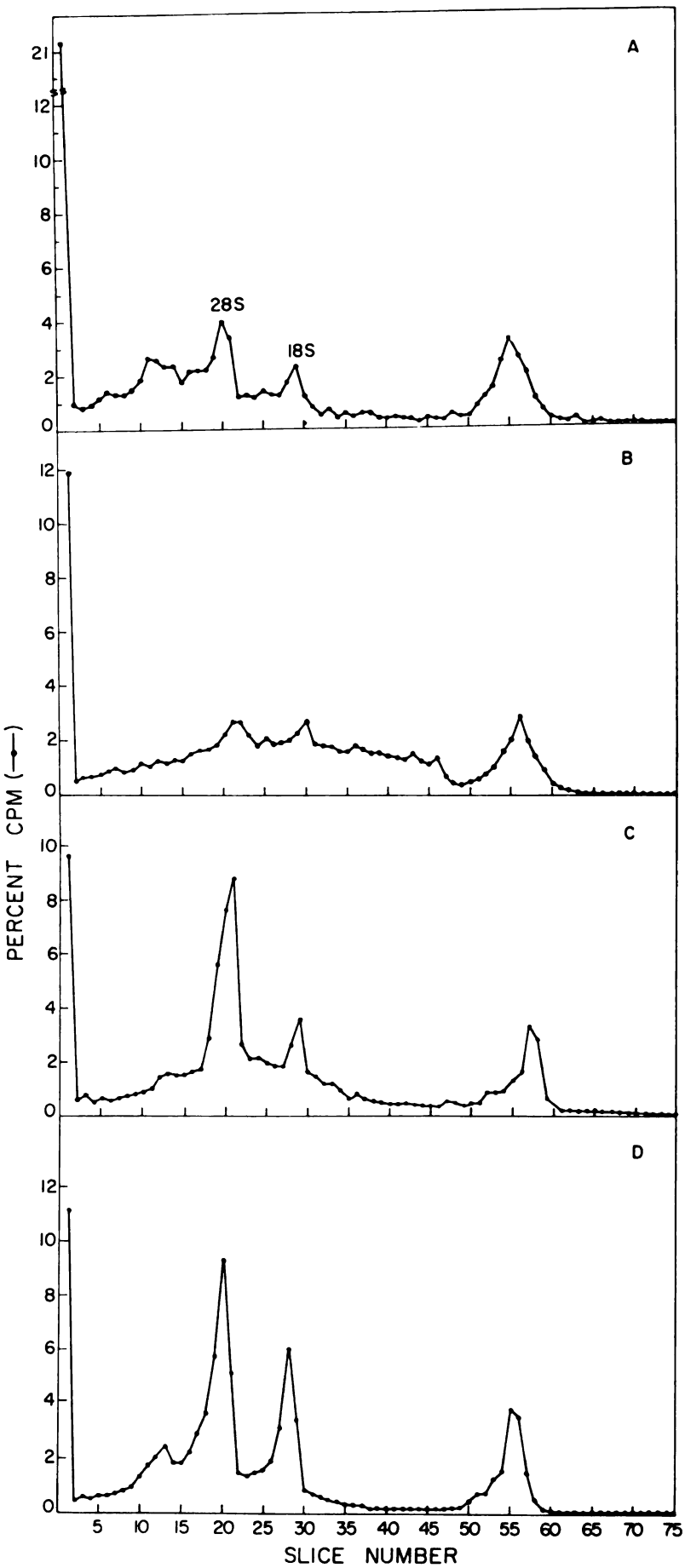

A
B
C
D
28S
18S
PERCENT CPM (—•—)
SLICE NUMBER
0
2
4
6
8
10
12
21
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75

Table 3. Percent of radioactivity in major RNA species in RNA from pancreatic rudiments labelled in vitro at pH 7.1.[a]

Incubation Period	Embryonic Age	28S %	18S %	5S, 4S %
4 hours	14-day[b]	14.2	9.2	19.3
	17-day[b]	14.8	11.4	24.2
	19-day	15.0	10.0	41.5
8 hours	14-day[b]	15.3	10.3	15.0
	17-day	41.0	19.3	21.4
	19-day	13.5	8.2	41.2
12 hours	14-day[b]	34.5	10.7	13.3
	17-day	44.8	19.4	18.2
	19-day	30.2	15.3	26.9
24 hours	14-day[b]	35.0	19.1	16.0
	17-day	53.5	20.4	14.3
	19-day	36.2	17.5	20.8

[a]Total cpm in gel slices of representative electropherograms were corrected for background counts. The counts in regions corresponding to 28S, 18S, and 5S and 4S are expressed as percent of the total counts.

[b]Because of the large percentage of counts at the origin, the first slice was not included in determining the percentage of total cpm.

described in the preceding section. Stained gels of RNA extracted from labelled tissue gave bands similar to 14-day pancreatic RNA on agarose-acrylamide gels (Figure 23). The distribution of label after 4 hours incubation was comparable to 14-day 4-hour radioactivity profile (Figure 24). Unlike 14-day pancreas, RNA from 17-day pancreatic rudiments continued less labelling appearing in material with a relative mobility greater than 28S rRNA after 4 hours culture. Radioactive profiles were comparable to absorbance scans were evident after 8 hours of culture. Variable amounts of DNA were extracted. The bread peak in the tRNA region of the radioactive profile suggest that some degradation occurred (Figure 24).

Analysis of RNA from 19-Day Old Pancreatic Rudiments

Nineteen-day old pancreatic rudiments were cultured as described in METHODS. Electrophoresis of labelled 19-day pancreatic RNA gave results similar to 14-day pancreas (Figure 25). Twenty-eight-S and 18S rRNA were detectable after 4 hours of culture. The distribution of label in 28S and 18S RNA matched the absorbance profile by 12 hours of culture.

The broad bands in the tRNA region and the material migrating between 18S and 5S rRNA indicate possibly more degradation than at earlier ages. Also, the counts

Figure 23. Photograph of RNA extracted from 17-day pancreas cultured for various intervals with [^{3}H] uridine at pH 7.1. RNA was extracted from 150-200 µg of tissue as described in METHODS. Slot 1 contained RNA from adult liver; slot 2, RNA from 17-day pancreas cultured for 4 hours; slot 3, 8 hours; slot 4, 12 hours and alot 5, 24 hours.

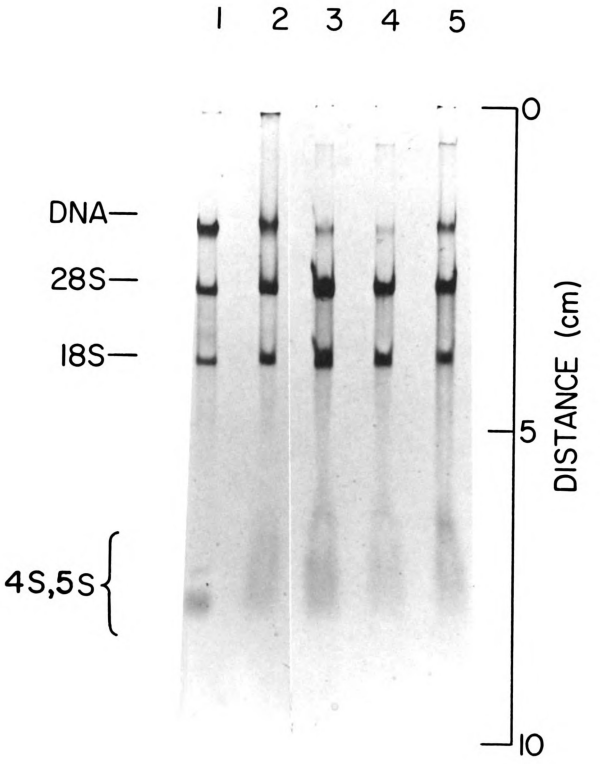

1
2
3
4
5
DNA
28S
18S
4S,5S
0
5
10
DISTANCE (cm)

Figure 24. Distribution of radioactivity in RNA from 17-day fetal rat pancreas cultured with [^{3}H] uridine at pH 7.1. Seventeen-day fetal pancreas was labelled and the RNA analyzed as described in Figure 20. Labelling times were: A, 4 hours; B, 8 hours; C, 12 hours, and 24 hours. (●——●) counts per minute, (----) absorbance.

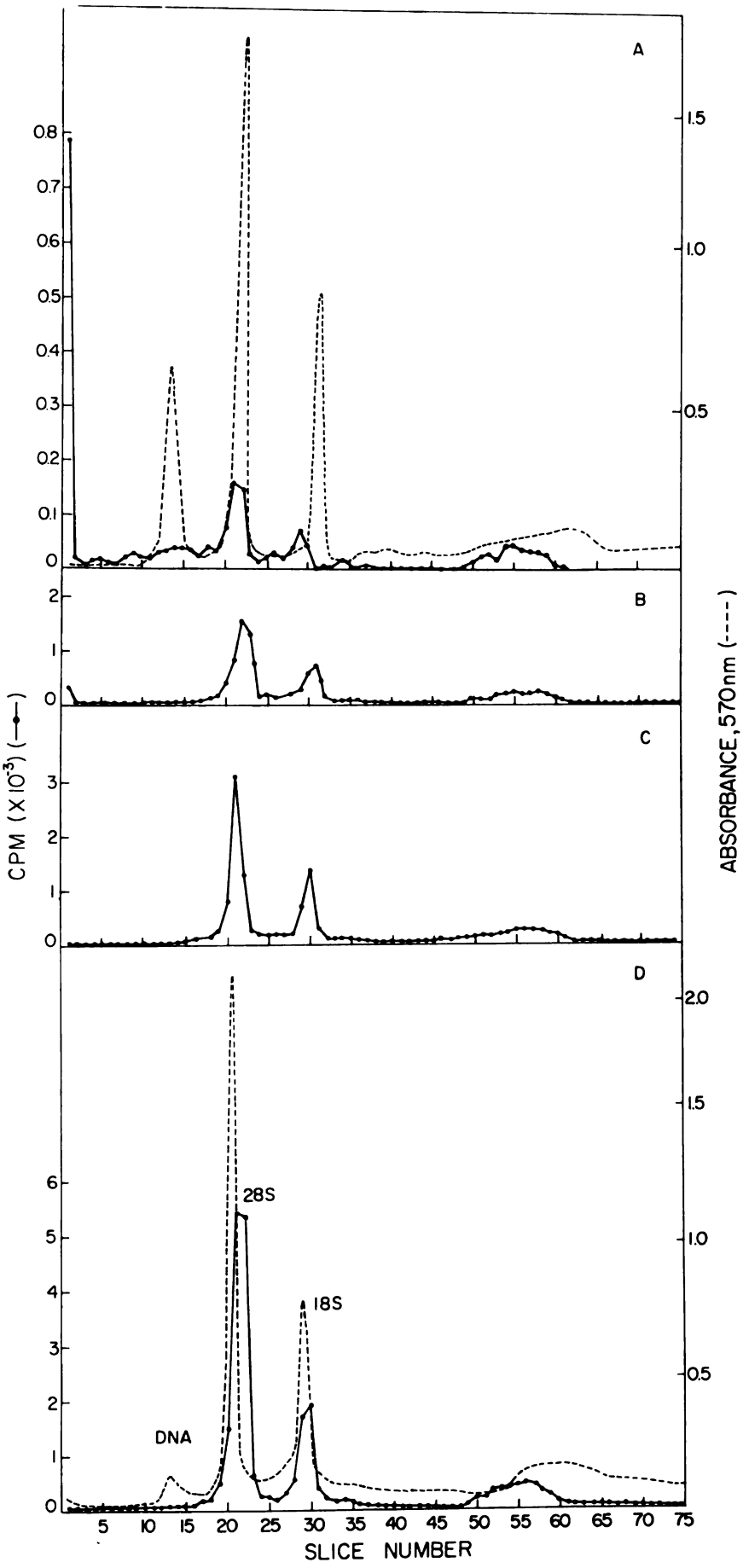
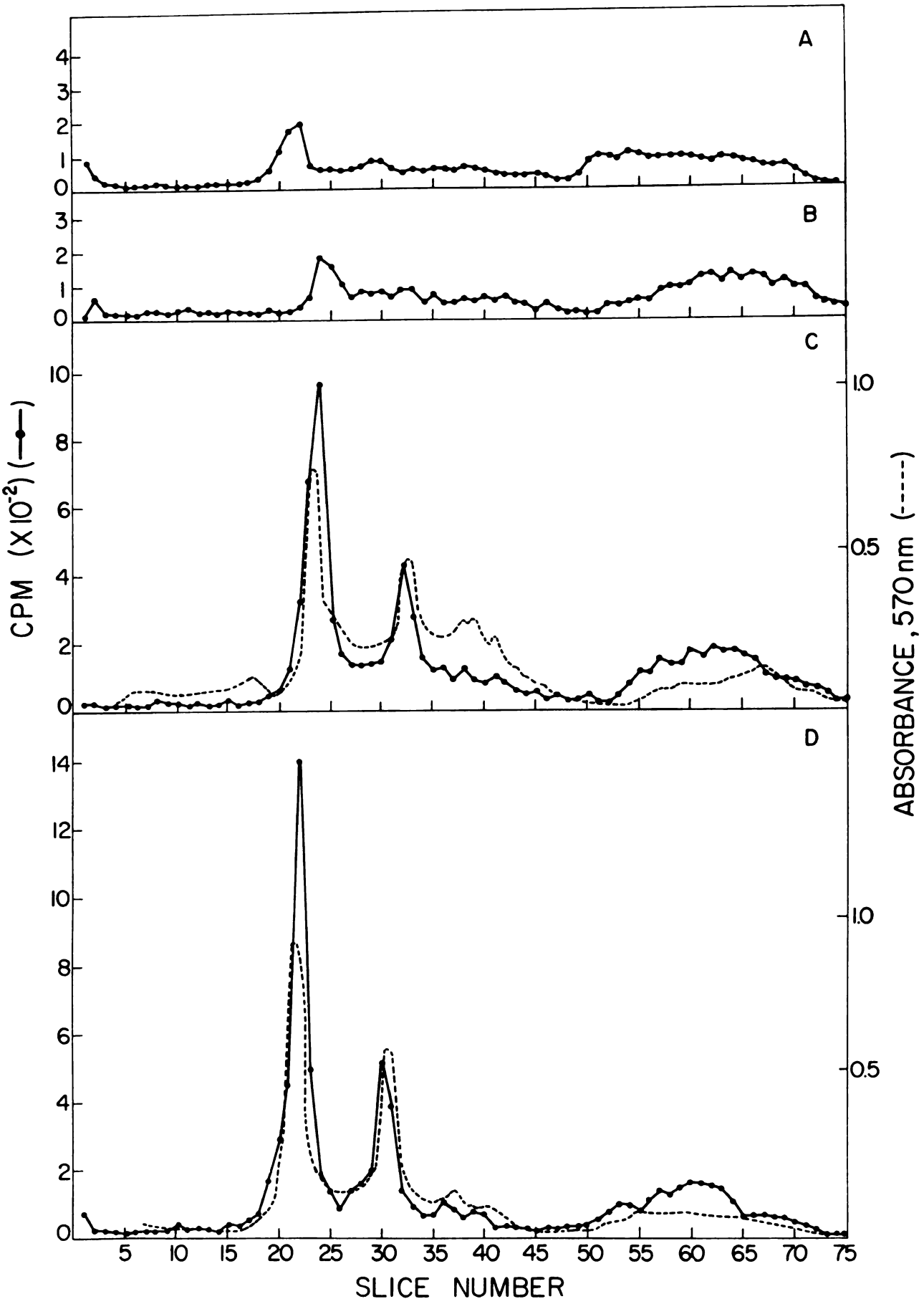

A
B
C
D
28S
18S
DNA
CPM (X 10⁻³) (—●—)
ABSORBANCE, 570nm (----)
SLICE NUMBER

Figure 25. Distribution of radioactivity in RNA from 19-day fetal rat pancreas cultured with [^{3}H] uridine at pH 7.1. Nineteen-day fetal pancreas was labelled and the RNA extracted as described in Figure 20. Labelling times were: A, 4 hours; B, 8 hours, 12 hours, and 24 hours. (●——●) counts per minute, (----) absorbance.


A
B
C
D
CPM (X10⁻²) (—●—)
ABSORBANCE, 570nm (-----)
SLICE NUMBER

migrating with an apparent molecular weight greater than 28S are absen even after 4 hours. This may be due to the greater degradation of RNA extracted from 19-day pancreatic rudiments or it may indicate a high molecular weight RNA species which is found in 14-day pancreatic rudiments but not in 19-day old rudiments.

Comparison of Distribution of Labelled 28S, 18S and 5S and 4S RNA

The radioactivity profiles from RNA extracted from 14, 17 and 19-day old pancreatic rudiment were used to compile the data in Table 3. For all ages, the percentage of counts in 28S and 18S increased from 4 to 12 hours, while the percentage in 5S and 4S decreased from 4 to 24 hours. The 19-day pancreas differed from other ages in that at 4 hours 41% of the label was in low molecular weight RNA. As the labelling time continued, the percentage dropped to 21%. Seventeen-day pancreas also synthesized a high percentage of low molecular weight RNA early and then the percentage of counts in that region gradually declined. There was also a variation in the percentage of counts in 28S and 18S. In Table 1 it was found that the usual 28S/18S ratio is 2, but after 4 hours at each age, the ratio of percent counts in 28S: percent counts in 18S was approximately 1.5. After 8 hours the ratio

was still less than 2 for 14 and 19-day old rudiments while 17-day old rudiments had a ratio of 2. By 12 to 24 hour of culture, the 28S/18S ratio was approximately 2 for all ages.

DISCUSSION

Extraction of RNA from Pancreatic Tissue

Because of the high concentration of ribonuclease present in the tissue, extraction of RNA from pancreas is very difficult. This study indicates that with low temperatures and rapid manipulations intact rRNA and tRNA can be extracted. Although undegraded RNA can be visualized on agarose-acrylamide gels, it is not possible to quantitate the extent of degradation. Minor degradation was assessed on gels by the presence of band between 28S and 18S, 18S and 5S, and a broad band of nucleotides migrating faster than 4S. Extensive degradation resulted in no distinguishable 28S and 18S band with a smear of stain throughout the gel. Similar characteristics were used by Green to assess degradation in RNA from mammary tissue.

It is highly probably that a small percentage of the RNA was degraded during extraction. Such a percentage could easily encompass minor RNA species such as mRNA, HnRNA and r RNA precursors. This would make their presence undetectable. The extraction of pancreatic RNA differed from such things as liver in that even with

precautions a substantial number of samples were lost due to degradation. However, the data presented here had no major degradation as judged by band migration, scans of stained gels or radioactive profiles of labelled RNA.

The extraction procedure used in this study was comparable to the procedure used by Green, et al (71), for the rapid extraction of undegraded RNA from cultured mammary tissue. Attempts to duplicate their exact protocol proved unsuccessful; only degraded RNA was extracted from pancreas. Their procedure entailed homogenization at 0°C in Tris-EDTA-borate buffer, pH 8.3, followed by phenol extraction. These two steps were found to be critical for pancreatic RNA extraction because degradation occurred between disruption of the tissue and addition of the phenol. By combining these steps into a single operation, undegraded RNA was extracted. The extracted RNA species observed on stained gels were 28S, 18S, 5S and 4S, with occasional DNA; these results were comparable to the profiles obtained by Green, et al (71) for differentiating mammary tissue.

The 400 µl extraction volume used by Green was not concentrated enough to analyze the small amount of RNA extracted from pancreatic rudiments. Consequently, the extraction volume was reduced to 100 µl. This allowed sensitive and qualitative analysis of small amounts of

RNA, but because of the small volume, quantitative analysis was not obtained. Large percentages of [^{3}H] uridine counts were lost in the extraction vessel and the protein at the interphase of the phenol and aqueous layers.

Method of Culturing Pancreatic Rudiments

Modifications were made in culturing pancreatic rudiments to allow easier handling of the small pieces of tissue. Usually, rat pancreatic rudiments are placed on Millipore filters anchored at the liquid-gas interphase of the tissue culture medium (2, 4). Other tissues have been cultured by floating a cellulose acetate raft on the surface of the tissue culture medium and placing the tissue on the raft (68). This method was modified for pancreatic rudiments in that the pieces of tissue were attached to a Millipore filter and floated tissue down on the surface of tissue culture medium. This allowed the entire tissue to be in contact with the labelled medium. By not anchoring the filter, the tissue could be readily transferred by merely picking up the support filter with sterile forceps.

Cytodifferentiation in pancreatic epithelia is usually studied by culturing 12 or 13-day old rudiments until they are 20 days old. This interval spans pancreatic differentiation and may entail up to 8 days in culture. During this period, the pancreatic rudiments differentiates,

but grows very little. The limited growth of the tissue suggests that the culture medium may lack some growth factor normally found *in vivo*. To alleviate this problem and to achieve rates of protein and RNA synthesis comparable to *in vivo* rate, pancreatic rudiments were excised at the desired embryonic age and cultured with [^{3}H] precursors for 24 hours. This resulted in a maximum rate of protein synthesis per microgram of protein about 2.4-fold greater than rates obtained from pancreatic rudiments differentiating *in vitro* (72). Rutter, *et al* (72) reported a 1.25-fold increase between 15 and 18-day rudiment. This study reports a 3-fold increase which is about the difference in the maximum and minimum rates per macrogram of protein throughout cytodifferentiation.

The method used to culture fetal pancreas was also found to be suitable for culturing adult pancreas. Uptake of [^{3}H] leucine and [^{3}H] uridine was linear for 24 hours at pH 6.8 and 7.1. By utilizing this technique, mechanisms regulating pancreatic secretion can be investigated. Membrane turnover can be studied under defined conditions and the effect of hormones and drugs on pancreatic metabolism can be noted.

Rates of Incorporation of [^{3}H] Leucine into Protein and [^{3}H] Uridine into RNA during Pancreatic Development

The uptake curves for leucine and uridine reflect the complexity of differentiation in mammalian systems. The incorporation kinetics for leucine and uridine varied from age to age and indicated a fluctuation in the rate at which internal precursor pools equilibrated. This is to be expected because each day, during differentiation, the tissue changes its internal structure and becomes more specialized than the day before. This change may occur suddenly as in sea urchin oogenesis. Consequently, a rudiment at a given embryonic age must be evaluated as a separate histological unit with its own set of metabolic characteristics. This is comparable to studying a different tissue each day.

Several of the difficulties involved in the uptake studies in organ culture can be attributed to the physical dimensions of the tissue. In cell suspension culture, all cells have equal access to the nutrients and labelled precursors in the medium, but in organ culture, the cells in the interior of the tissue are isolated from direct contact with the culture medium. All nutrients must diffuse into the tissue and waste products must diffuse out. Cells near the surface could be labelled more heavily with an isotope than cells in the center. The

problem was resolved in this study by cutting the tissue into very small pieces of 5 to 15 μg, a size which eliminated diffusion as the rate limiting step. But even so, statistical variations in the size of the tissue pieces did occur and probably added to the complexity of the data.

Further complexity is derived from the cellular composition of the developing pancreas. The adult pancreas is known to contain a variety of cell types which range from the secretory acinar and beta cells to the fibrocytes in the connective stroma. During pancreatic differentiation, the ratio of these cells is constantly changing. Around the 14-day of gestation, beta cells differentiation is complete and connective tissue is abundant. Later as the acinar cells proliferate and differentiate, their percentage increases. It is conceivable that nonsecretory cells as fibrocytes could make major contributions to macromolecular synthesis. This study suggests that to be unlikely. If the increase in RNA synthesis observed were due to nonsecretory cells, the rates would have decreased linearly with age as the percentage of secretory cells increased and nonsecretory cells decreased. Because linear kinetics were not shown by the apparent rate curves, it is unlikely that differences in precursor uptake were due to ratio changes in the cell population of the tissue. The nonlinearity of the

data suggests that other factors besides secretory cell proliferation were influencing macromolecular synthesis.

Another possible complication is the fact that the pancreas develops in two parts, the dorsal and ventral lobes, which merge around 16 and 17 days to become more like the adult organ. Of the two lobes, the dorsal lobe is the larger and the most likely piece to be dissected out of embryos younger than 16 days. There may be a difference in the synthetic activities of the dorsal lobe and total pancreas which would cause studies of younger and older tissues to be incompatible. This was partially resolved by Spooner, et al., who showed the close similarity of development in dorsal and ventral pancreases (6). However, it is still possible that the merging of the two lobes had some effect on the rates of precursor incorporation.

The study was designed to measure transitions in the rates of RNA synthesis during cytodifferentiation. In the rat pancreas, cytodifferentiation occurs between 14 days and 20 days of gestation. The primary events which initiate differentiation occur at an earlier age. At the start of cytodifferentiation the epithelial cells are small and have very little rough endoplasmic reticulum, a small Golgi apparatus and no zymogen granules. After differentiation, they have an extensive rough endoplasmic reticulum, an enlarged Golgi apparatus and numerous zymogen

granules. The increase in rough endoplasmic reticulum with the corresponding increase in ribosomes during cytodifferentiation suggest a transition in the rate of RNA metabolism in the cell. This may occur as a change in RNA synthesis, or a change in RNA turnover. This study focused on changes in RNA synthesis. Because data were available on [^{3}H] leucine incorporation by fetal rat pancreases in culture (73), leucine uptake was used as a control to access tissue survival in culture.

In evaluating the apparent synthetic rate curves at pH 7.1, a maximum rate per cell is seen in RNA synthesis on day 18 followed by a maximum rate in protein synthesis on day 19. This is in accord with the sequence of transcription and translation in that a change in RNA synthesis would be expected to precede a change in protein synthesis. This suggests that RNA synthesis is the rate limiting step in protein synthesis. The major transition in the rates of RNA and protein synthesis paralleled the rates of synthesis of marker enzymes measured by Rutter, et al. (2, 72). Both studies showed a gradual increase in the rates of protein synthesis as the pancreas became more specialized. However, in this study the change in macromolecular synthesis could have been due to a decrease in the size of the soluble pool used for immediate synthesis. This possibility was not explored in the study

because of the technical problems involved in obtaining enough fetal pancreas for pool analysis.

Other researchers have reported changes in RNA synthesis during hormone induced cytodifferentiation. Chicks given daily injections of estradiol and then withdrawn from treatment had a reduction in RNA and protein synthesis in the tubular gland of the oviduct. If estradiol or progesterone were then administered, protein and RNA synthesis were reestablished with a several-hundred-fold increase in the rate of ovalbumin synthesis (74). Estrogenic hormones also stimulate the synthesis of tRNA in chick oviduct (75). RNA synthesis in rat mammary gland in culture was stimulated by prolactin. Addition of hydrocortisone and insulin caused the non-secretory alveolar cells of the mammary gland to differentiate into secretory cells (76). These studies support the tentative conclusion that the rate of RNA synthesis increases during cytodifferentiation in fetal pancreases.

Changing the pH of the culture medium from 6.8 to 7.1 had little effect on the rate of RNA synthesis, and yet the radioactive profiles of the labelled RNA on gels were distinctly different. This suggests that the lower pH effected the RNA species being synthesized by the pancreatic rudiments. In contrast, the rates of protein synthesis were drastically affected by the pH change. It is possible that the pH effect on protein synthesis was

the result of an impaired RNA synthesizing mechanism which reduced the synthesis of a rate-liminting RNA molecule.

Electrophoretic Analysis of [^{3}H] RNA

The difference in the percentage of the absorbance and the percentage of radioactivity for each major species was due to the method by which each was determined. The absorbance percentages were based on the total area under each major RNA peak, instead of the total absorbance above the baseline. The radioactivity percentages were based on the total amount of radioactivity in each slice. Consequently, the percentages varied between the two tables, but the ratios of the major RNA species are comparable and they provide a valid means of comparison.

The analysis of [^{3}H] uridine RNA from 14, 17 and 19-day pancreases cultured at pH 7.1 indicates that there is no measurable difference in the percentage of 28S and 18S synthesized during cytodifferentiation as judged by 24-hour labelling experiments. An increase in the percentage of radioactivity in 4S RNA with increasing age suggests that more tRNA is synthesized by the 19-day pancreases than by the younger 14 and 17-day tissue. This increase, however, coincides with an increase in ribonuclease and could be an artifact of degradation during extraction. A comparison of the percentage of 4S RNA in

19-day pancreatic rudiments shows a decrease in 4S RNA as more 28S and 18S RNA is labelled. If the increase in radioactivity in 4S RNA throughout cytodifferentiaion were due to the higher ribonuclease content of older tissue, the percentage of label in the 4S peak would have increased in proportion with the increased labelling of 28S and 18S RNA. It is probable that degradation contributed to the percentage of label in the 4S peak, but the contribution is negligible when compared with the percentage decrease throughout the time course. This is in agreement with the reported increase in tRNA synthesis during hormone-induced differentiation in chick oviduct (75).

A larger percentage of counts from 14-day old rudiments migrated on gels with an apparent molecular weight greater than 28S. Green, et al. also reported a large high percentage of count migrating in the high molecular weight region of the gel when mammary gland RNA was labelled for 2 to 4 hours. The two observations differ in that very few counts migrated in this region when RNA was extracted from mammary glands labelled for 24 hours; RNA from 14-day pancreatic rudiments labelled for this period still had a large percentage of counts in the high molecular weight region of the gel. The variation could be explained by a difference in DNA synthesis between the two tissues. Fourteen-day pancreatic rudiments contain rapidly dividing cells as well as

differentiating cells. In the rapidly dividing cells of 14-day pancreatic rudiments, DNA synthesis occurs in hours, while in mammary gland DNA synthesis occurs in days (76). After labelling periods of 2 to 4 hours, it is likely that a large percentage of count are in high molecular weight RNA. In 14-day old pancreases, these transient RNA molecules may be replaced by DNA. This also explains the absence of radioactivity in this region of the gel when labelled RNA is extracted from older rudiments. Seventeen and 19-day old rudiments have fewer rapidly dividing cells than 14-day old rudiments, so, the extent of DNA labelling in these tissues may be comparable to mammary gland. Also, the older rudiments contain increasingly high concentrations of ribonuclease which could have degraded any high molecular weight RNA labelled in 4 hours. This would explain the absence of labelled high molecular weight component in 17 and 19-day rudiments labelled for 4 and 8 hours. A third possible explanation is that 14-day old rudiments contain a high molecular weight species of RNA not found in older more differentiated tissue with the 4-hour labelling period.

Significance

This study defines 24-hour culture conditions for fetal pancreases which allow rapid and easy handling of small tissue pieces. The 24-hour incubation gave

incorporation rates for [^{3}H] leucine which were approximately 4-fold greater than rates obtained from in vitro cytodifferentiation of rat pancreases. The sensitivity of slab gel electrophoresis was increased to allow fast analysis of small quantities of RNA. With these two procedures, the methionine dependent differentiation of rat pancreas and its effect on RNA methylation can be investigated. Because of the high ribonuclease content of older pancreatic rudiments, it is suggested that any further RNA studies be limited to rudiments which are younger than 17 days old.

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