Effect of Hydrocortisone on Chick Duodenum Cultured in Chemically Defined Medium* (34118)

JACQUELINE C. HIJMANS AND KENNETH S. MCCARTY

Departments of Medicine and Biochemistry, Duke University School of Medicine, Durham, North Carolina 27706

Previous experiments have demonstrated that the specific activity of invertase in cultures of the duodenum from 16- and 19-day-old chick embryos was stimulated by hydrocortisone in a medium enriched with calf serum (1). Since Olmsted has shown that several factors in fetal calf serum may be toxic for the growth of certain cells in culture (2), and because of our desire to study glucose and amino acid utilization by the cultured duodenum, it became necessary to study cultures of this organ in a chemically defined medium. The results of these experiments are presented in this paper.

Methods. Sixteen-day-old embryonic duodenum was cultured in organ culture dishes with a 20 × 2-mm center well and an absorbant ring as previously described (3). For 19-day-old tissue, however, it was necessary to use larger (35 × 10 mm) culture dishes to insure sufficient medium. The Millipore membranes were supported on a Teflon ring. Preparation of the cultures was otherwise identical and has been described previously (1). Duodenal fragments were distributed at random among four dishes, with the serosal side in contact with the Millipore membrane. Two cultures contained medium to which 10% calf serum was added and two cultures contained serum-free medium. Of each set of two dishes, one contained 0.5 μg of hydrocortisone per ml medium. The fragments (1–2 mm) were evenly distributed to assure that the serosal side maintained intimate contact with the Millipore membranes. All cultures were incubated in a 5% CO_2-air humidified chamber at 37°. After 3 days of incubation, the Millipore membranes with the tissue fragments were rinsed twice in iced saline. The tissue was then collected, quickly frozen, and stored. The serum-free medium on which the fragments were cultured was collected at the same time. Two dishes with serum-free medium in which no tissue was cultured were incubated simultaneously and for the same length of time and served as controls.

Procedures. The tissues were homogenized and the homogenate prepared for invertase and protein determinations as previously described (1). Invertase determinations on 0.4-ml aliquots were made according to the procedure of Dahlqvist (4) using glucose oxidase (Glucostat special reagent, free of invertase), to measure the amount of glucose released. Zero time blank values and glucose released in the absence of substrate were subtracted as controls for glucose in the tissue. Proteins were precipitated by the Somogyi procedure (5). Specific activities were expressed as micrograms of glucose released per milligram of protein per hour. Protein determinations were made according to the procedure of Lowry *et al.* (6) using 0.05–0.1-ml aliquots, with bovine serum albumin as a standard. The glucose oxidase reaction was also used to determine the amount of free glucose in the medium (2% accuracy). One-half milliliter of medium was centrifuged for 10 min at 900*g*. An aliquot of 0.05 ml from the supernatant portion was used for the glucose determination. An equal aliquot of the control medium served as the standard. Amino acids were determined as described previously (7).

Materials. Millipore (R) filters (0.45 μ)

* Supported in part by USPHS Grant, NIH GM 12805-04 and the American Cancer Society Grant P363D and by USPHS Grant, RO1-H002838 and a grant from the Duke University Research Council.

were obtained from the Millipore Corporation. Brij 35: Pierce Chemical Co., Rockford, Ill. Glucostat Special: Worthington Biochemical Corp., Freehold, N.J. Tris buffer (Tris-Trizma Base): Sigma Chemical Co., St. Louis, Mo. Sucrose: M.A. Special Enzymatic Grade, Mann Research Laboratories, Inc., New York. Iodoacetic acid: Fluka, A.G., Buchs, S.G., Switzerland. Organ culture dishes were obtained from Falcon Plastics. Solu-Cortef (Hhydrocortisone-sodium hemisuccinate): Upjohn Co., Kalamazoo, Mich. Tissues were cultured in Eagle's medium modified by supplementation with glucose to give a final concentration of 200 mg/100 ml of medium. Embryonated Plymouth Rock–White Rock chicken eggs were obtained from the Central Carolina Farmers' Exchange, Durham, N.C. The eggs were placed in a 37° humidified incubator until the desired day of the experiment. Beak and toe measurements were used to verify embryonic age (8).

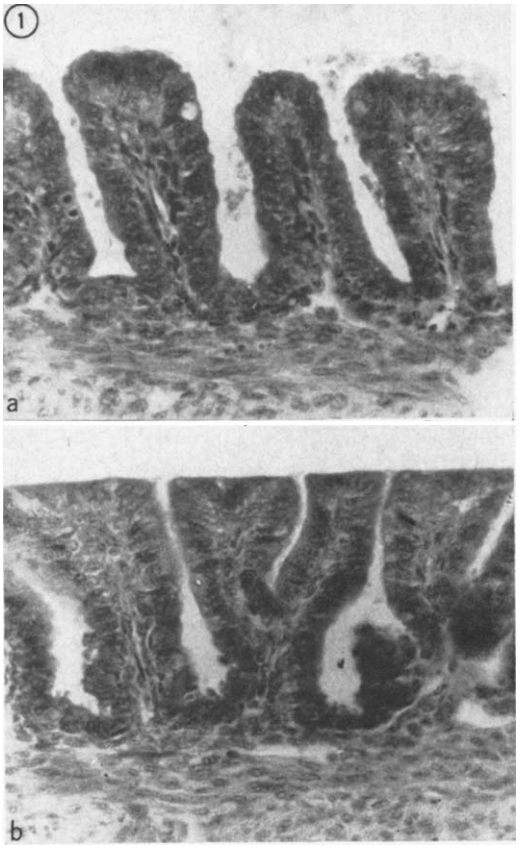

FIG. 1. Photomicrographs of Bouin-fixed, paraffin-embedded sections of cultured 16-day embryonic chick duodenum. Sections were stained with hematoxylin-eosin. A. Villi of 16-day chick embryo duodenum cultured for 3 days in calf serum–enriched medium. B. Villi of 16-day chick embryo duodenum cultured for 3 days in a chemically defined medium.

TABLE I. Number of Mitoses Seen per 100 Villi.[a]

	Without hydrocortisone	With hydrocortisone
With serum	18	22
Without serum	19	13

[a] Each figure represents the average number of mitoses seen in 900–1000 villi in a total of four different experiments.

Results. Microscopic observations. The cultured tissue fragments were sectioned and stained as reported previously (9). No significant differences in appearance by light microscopy could be seen in the tissues after culture for 3 days in the presence or absence of serum (Fig. 1A, B). Surface epithelial cell mitoses were counted in duodenal fragments of 16-day embryos. Every fifth section of serially cut tissue was used to count the cells and only well oriented villi which were sectioned along the entire length were examined (9). These studies indicate that serum in the absence of hydrocortisone did not exert any effect on the mitotic index of the duodenal fragments after 3 days in culture (Table I). However, serum relieved the inhibition by hydrocortisone. The mitotic cells in all cultures were found predominantly (78-83%) in the crypts and lower half of the villi.

Invertase specific activity. Invertase specific activity was determined in duodenal fragments of 16- and 19-day-old embryos cultured for 3 days in media with and without serum. It is necessary in these experiments to provide adequate control for the variability in invertase activity from tissues obtained from different embryos. Therefore explants from the same embryos were used for both the control and the experiment. The average invertase specific activity of 16-day explants cultured in serum-containing medium was 48 as contrasted to 64 in medium without serum

TABLE II. Effect of Serum on Invertase Activity in Chick Duodenum.

	Medium with serum[a]						Medium without serum					
	Without HCT[b]			With HCT			Without HCT			With HCT		
Expt.	TP	TI	ISA[c]	TP	TI	ISA	TP	TI	ISA	TP	TI	ISA
16-Day explants												
1	1.9	107	55	1.7	186	110	1.7	146	84	1.8	278	152
2	1.7	83	48	2.1	151	73	1.4	67	48	2.0	179	91
3	1.6	107	69	1.8	161	90	1.8	102	58	1.7	172	100
4	1.6	33	21	1.3	74	56	1.4	51	38	1.2	106	92
5	1.6	69	42	1.7	83	50	1.6	83	53	1.7	179	103
6	1.1	41	39	1.1	103	97	0.7	59	91	1.1	97	91
7	0.6	44	91	0.9	98	112	0.9	65	70	0.6	73	127
8	1.1	22	21	1.2	69	60	0.9	60	70	1.0	88	93
Average			48			81			64			106
19-Day explants												
1	1.4	66	47	1.5	118	80	1.3	98	77	1.1	96	91
2	2.0	152	76	2.1	251	122	1.6	171	105	1.4	192	134
3	1.3	101	80	1.2	161	130	1.4	165	119	1.4	200	146
4	1.2	111	95	1.3	183	137	0.9	132	140	1.2	183	155
5	1.2	97	73	1.1	150	127	1.3	213	153	1.0	172	165
6	1.7	183	109	1.4	129	94	1.3	200	158	1.4	259	180
Average			80			115			125			144

HCT = hydrocortisone; TP = total protein; TI = total invertase; ISA = invertase specific activity.

[a] 10% calf serum, not dialyzed.

[b] Hydrocortisone; 0.5 μg/ml medium, cultured for 3 days.

[c] Invertase specific activity expressed as μg of glucose released per mg of protein per hour.

In each set of experiments, duodenums of eight embryos were used in 16-day explants and of 4–5 embryos in 19-day explants.

(Table II), a 34% increase in invertase activity when serum was omitted from the medium. The results presented in Table II demonstrate that hydrocortisone is effective in stimulating invertase activity in the presence or absence of serum for both the 16- and 19-day explants.

Analysis of the medium. Glucose utilization, Table III. Consumption of glucose from the medium by the duodenum of 16-day-old embryos during 3 days of culture without hydrocortisone averaged 1.3 mg of glucose per mg of cell protein. When hydrocortisone was added to the medium, the consumption for the 3-day period was decreased to an average value of 0.8 mg. Thus there was 35% less uptake of glucose by the tissues when cultured in the presence of hydrocortisone.

The decreased uptake seen with hydrocortisone in the 16-day-old tissues was not found in the case of the 19-day-old tissues. Corresponding figures for the 19-day-old duodenum cultures were 1.6 and 1.6 mg of glucose per mg of cell protein (Table III).

Amino acid utilization. In agreement with previous observations on amino acid metabolism of established cell strains, (7) the duodenal tissue cultures (Table IV) demonstrated both a selective utilization of essential as well as the production of nonessential amino acids. It is of interest that one difference between 16-day explants and 19-day explants is rejected in the preferential utilization of histidine and arginine by the 16-day tissues whereas the older explants utilized more isoleucine and leucine. The ratio of essential amino acids utilized to nonessential amino acids produced and lost to the medium was

TABLE III. Effect of Hydrocortisone on Glucose Consumption.

Experiment	Without hydrocortisone (mg glucose/mg protein)	With hydrocortisone (mg glucose/mg protein)
16-Day explants[a]		
1	1.4	1.3
2	1.8	1.6
3	.8	.4
4	.7	.7
5	1.5	.6
6	1.1	.5
7	1.2	.6
8	1.5	1.2
9	.9	.5
10	2.0	1.0
Average	1.3	0.8
19-Day explants[a]		
1	1.5	1.8
2	1.6	1.5
3	2.0	1.6
4	1.7	2.0
5	1.9	2.0
6	1.7	2.3
7	1.9	1.4
8	1.4	1.1
9	.9	1.1
Average	1.6	1.6

[a] Chick embryonic duodenum cultured for 3 days in a chemically defined medium.

greater for 16-day explants than 19-day explants. This ratio for the controls of the 16-day explants was 2.07 and 2.14 and for the 19-day explants 1.13 and 1.29. With hydrocortisone added to the medium the ratio was 2.08 and 2.14 for the 16-day explants and 1.25 and 1.22 for the 19-day tissues.

Although hydrocortisone did not appear to exert any influence on the utilization of individual amino acids in either 16- or 19-day explants, the ammonia released by the 16-day explants was markedly decreased in the presence of the hormone. This effect was not observed in the 19-day explants (Table IV).

Discussion. Olmsted (2) pointed out that different batches of fetal calf serum had variable effects on tissue cultures and that certain lots were apparently toxic. Since this variability might complicate the interpretation of experiments using organs cultured in the presence of serum, it became necessary to utilize a serum-free medium as has been done with other tissues (10, 11, 13). Additionally, the analysis of the effects of various inducing agents; *e.g.*, hydrocortisone, on the metabolism of the cultured tissues would be greatly improved if the chick embryo duodenum could be maintained in a serum-free chemically defined system.

No significant morphologic differences were ascertained from the histologic sections of chick embryo duodenum cultured for 3 days without serum as compared to the tissues grown in the presence of serum (Fig. 1A,B). Cells in various stages of mitosis were present in all cultures. The location of the mitotic figures indicated that the tissues underwent maturation as well as growth in the absence of serum. In the 16-day-old duodenum, mitotic figures normally are present throughout the surface epithelium, both on the villi as well as in the crypts. In the normal 19-day-old embryonic duodenum, mitotic figures tend to be predominant in the crypts and in the lower half of the villi. This distribution was seen in the 16-day-old duodenum which had been cultured for 3 days in the absence of serum.

Invertase specific activity was found to be higher in duodenal fragments cultured in the absence of calf serum than in its presence. Despite this increase, stimulation of the enzyme specific activity was induced to a greater extent by hydrocortisone in the absence of serum. Similar effects, but to a lesser degree, were seen in the cultured 19-day-old duodenums. As described previously, the effect of hydrocortisone was much less marked at this age (3).

It is fortunate that the induction of invertase activity by hydrocortisone may be elicited in either the presence or absence of serum, thus permitting a more detailed study of the effect of this hormone on glucose and amino acid metabolism.

The apparent decrease in glucose utilization caused by hydrocortisone in the 16-day duodenum may be explained by alterations in glycolysis, gluconeogenesis, or glycogenolysis in these tissues. Further studies are needed

TABLE IV. Effect of Hydrocortisone on the Amino Acid Utilization in Chemically Defined Medium.

	Amino acid utilization (μM/mg cell protein/3 days)							
	16-Day explants[a]				19-Day explants[a]			
	Expt 1		Expt 2		Expt 3		Expt 4	
Amino acid	With HCT[b]	Without HCT	With HCT	Without HCT	With HCT	Without HCT	With HCT	Without HCT
Threo	.12	.22	.18	.26	.11	.19	.12	.17
Glu NH_4	.33	.38	.31	.42	.40	.41	.42	.43
Val	.16	.19	.18	.21	.24	.23	.24	.25
Met	.01	.01	.01	.03	.09	.08	.05	.07
Iso	.36	.31	.35	.32	.40	.40	.43	.35
Leu	.27	.25	.28	.30	.47	.44	.45	.40
Tyro	.03	.02	.03	.05	.02	.03	.03	.02
Phe	.01	.01	.02	.06	.08	.07	.03	.03
Lys	.11	.05	.05	.11	.05	.05	.03	.01
His	.14	.19	.23	.22	.10	.01	.01	.01
Arg	.24	.20	.23	.26	.10	.06	.06	.06
Cys A.	+.10	+.10	+.18	+.24	+.16	+.04	+.06	+.09
Pro	+.13	+.14	+.13	+.23	+.30	+.40	+.37	+.32
Gly	+.09	+.07	+.08	+.03	+.07	+.06	+.04	+.05
Ala	+.48	+.51	+.44	+.50	+.01	+1.22	+1.03	+.93
Orn	+.05	+.07	+.05	+.08	+.03	+.02	+.02	+.03
NH_4	+.06	+.53	+.19	+.40	+.85	+1.17	+.88	+.87
Total utilized	1.78	1.85	1.89	2.24	1.96	1.96	1.86	1.80
Total produced	.85	.89	.88	1.05	1.57	1.73	1.51	1.40
Ratio U/P	2.08	2.07	2.14	2.14	1.25	1.13	1.22	1.29

[a] Chick embryonic duodenum cultured for 3 days.

[b] HCT = hydrocortisone.

to clarify this point. This phenomenon was not observed in the 19-day explants, possibly due to the fact that during this period (16–19 days), the duodenum undergoes considerable maturation. Moog and Richardson (12) suggested that functional differentiation of the duodenum is controlled by the adrenals of the embryo. Further, Watterson *et al.* hypophysectomized chick embryos and noted a delay in duodenal maturation, presumably through lack of pituitary–adrenal cortex interaction. The duodenal epithelium in embryos of hypophysectomized embryos never became columnar and there was deficient formation of alkaline phosphatase. He concluded that the differentiation of the duodenum is controlled by the chick's adrenals beginning about 16 days of age (13). Such prior stimulation by the adrenals *in vivo* might well explain the failure of cortisone to alter glucose consumption and the decreased stimulation of invertase activity by added hydrocortisone in the 19-day-old tissue.

In agreement with previous studies (7) there is a selective utilization of amino acids in cell cultures. The ratio of amino acids utilized to the amino acids synthesized was constant in the presence or absence of hydrocortisone in spite of the fact that this ratio was higher in younger explants. The small increase in amino acid utilization in the 16-day embryo in the absence of hydrocortisone is primarily the result of threonine utilization. The significance of this and the fact that less ammonia was produced in the presence of the hormone in the 16-day explant will require further investigation. It is possible to postulate, based on experiments

with liver cells, that glycogen breakdown may be stimulated by hydrocortisone and it will be of importance to investigate in some detail the sparing effect on glucose utilization and ammonia production. In addition, one can postulate that hydrocortisone also induces glycogen utilization and the deamination of amino acids with the concomitant formation of α-keto acids for the Krebs cycle function, thereby decreasing the glucose requirement in these cultures.

Summary. Sixteen- and 19-day-old embryonic chick duodenum can be cultured for at least 3 days in a chemically defined serum-free medium.

No significant morphologic difference is observed whether or not serum is added to the medium.

Cells in mitosis are present in all cultures and the distribution of mitoses indicates mat uration of the tissue in culture with or without serum.

Invertase activity is increased in cultures without serum and the induction of invertase by hydrocortisone is greater in tissues cultured in serum-free medium.

The utilization of glucose by the duodenum in organ culture is decreased in the presence of hydrocortisone.

The utilization or production of the individual amino acids by the chick embryo duodenum in organ culture in the presence and absence of hydrocortisone has been described.

The authors express their appreciation for the valuable technical assistance in this work performed by Mrs. Janet B. Barrett and Mr. Oliver E. Brown.

1. Hijmans, J. C. and McCarty, K. S., Proc. Soc. Exptl. Biol. Med. **123**, 36 (1966).

2. Olmsted, C. A., Exptl. Cell Res. **48**, 283 (1967).

3. McCarty, K. S., Hijmans, J. C., and Wilhelm, J. A., Ann. N. Y. Acad. Sci. **139**, 214 (1966).

4. Dahlqvist, A., Anal. Biochem. **7**, 18 (1964).

5. Somogyi, M., J. Biol. Chem. **160**, 69 (1945).

6. Lowry, O. H., Rosebrough, H., Farr, A. L., and Randall, R. J., J. Biol. Chem. **193**, 265 (1951).

7. McCarty, K. S., Exptl. Cell Res. **27**, 230 (1962).

8. Hamburger, V. and Hamilton, H. L., J. Morphol. **88**, 49 (1951).

9. Dobbins, W. O., III, Hijmans, J. C., and McCarty, K. S., Gastroenterology **53**, 557 (1967).

10. Trowel, O. A., Exptl. Cell Res. **16**, 118 (1959).

11. Biggers, J. D., Gwatkins, R. B. L., and Heyner, S., Exptl. Cell Res. **25**, 41 (1961).

12. Moog, F. and Richardson, D., J. Exptl. Zool. **130**, 29 (1955).

13. Watterson, R. L., Brown, D. A., and Bartha, A., Anat. Record **133**, 347 (1959).

Received Feb. 27, 1969, P.S.E.B.M., 1969. Vol. 131.