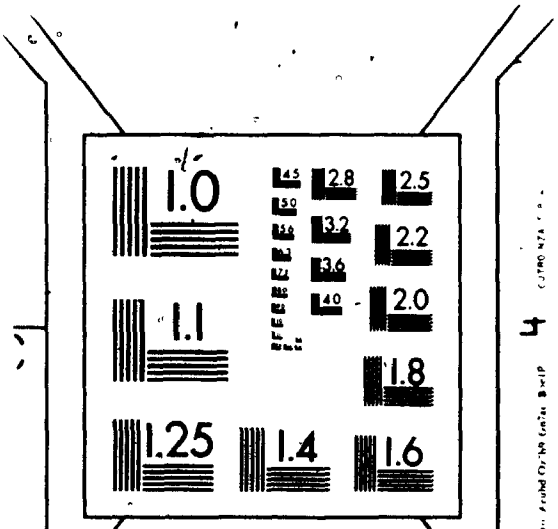
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NL-339 (3/77)

EFFECTS OF THYROXINE ON DUODENAL DEVELOPMENT IN CHICKEN EMBRYOS

by

LINDA M. BREWER, M.Sc.

A thesis submitted to the Faculty of
Graduate Studies in partial fulfilment
of the requirements for the degree of
Doctor of Philosophy

Department of Biology
Carleton University
Ottawa, Ontario
January, 1979

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The undersigned recommend to the Faculty of Graduate Studies acceptance of the thesis "Effects of Thyroxine on Duodenal Development in Chicken Embryos" submitted by Linda Marie Brewer, M.Sc., in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

.....
Thesis Supervisor

.....
Chairman, Department of Biology

.....
External Examiner

Carleton University
January 19, 1979

ABSTRACT

The role of thyroxine (T_4) in duodenal development in chicken embryos was studied through 1) treatment of embryos at 16.5 days of development with a single dose (5.7 μg pentahydrate salt) of L-T_4 and 2) the culture of stage 42 duodenal explants *in vitro* using synthetic medium (McCoy's 5A) containing L-T_4 (14 ng/ml or 14 ng/100 ml). In both sets of experiments D-T_4 was used as a physicochemical control.

In the experiments performed *in vivo*, 48 hours after treatment with L-T_4 duodenal alkaline phosphatase specific activity of stage 44 embryos was significantly greater than in all stage 44 control groups (intact, windowed, saline-treated, and D-T_4 treated). At 72 hours, the phosphatase activities of the control stage 45 groups had attained the level reached 24 hours previously with L-T_4 treatment, and the phosphatase activity of the stage 45 L-T_4 treated group was further elevated. Therefore treatment with L-T_4 produced elevation in duodenal alkaline phosphatase 24 hours precociously. When the results were pooled according to time (i.e. embryos of different stages pooled according to time elapsed since treatment), phosphatase remained significantly elevated 48 hours after L-T_4 treatment, but was no longer elevated at 72 hours.

Twenty-four hours after treatment, total duodenal protein content was significantly elevated in the stage 43 L-T_4 treated group, compared to all other groups except the D-T_4 treated one. In embryos treated with D-T_4 total duodenal protein content was slightly elevated.

On ultrastructural examination, the columnar absorptive cells of the duodenal epithelium showed increased Golgi activity and secondary lysosomes 48 and 72 hours after L-T_4 treatment in stage 44 and 45 embryos respectively. Glycogen and lipid droplets present in the absorptive cells disappeared more rapidly following L-T_4 treatment.

Treatment with L-T_4 also accelerated yolk sac retraction and produced a slightly greater incidence of pipping of the shell (72 hr) but did not otherwise accelerate overall embryonic development.

Following 48 hours of culture <u>in vitro</u>, with medium containing L-T_4 (14 ng/100 ml), the alkaline phosphatase specific activity of duodenal quarters was significantly elevated compared to explants cultured with 14 ng L-T_4/ml, D-T_4, or without hormone. In a similar set of cultures with bovine serum albumen added to all media, phosphatase was elevated in the presence of 14 ng L-T_4/ml. In both sets of cultures total duodenal protein was significantly elevated in explants cultured in the presence of D-T_4.

The morphology of the duodenal epithelial cells following culture ranged from normal to highly condensed in the appearance of the cytoplasm and organelles. These changes could not be attributed to the specific treatments, and were considered due to general effects of culture and or fixation. Goblet cell maturation proceeded more quickly in culture.

It is suggested that thyroxine normally participates in duodenal development in chicken embryos, and may do so through direct action on the epithelial cells.

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TABLE OF CONTENTS

	Page
List of Tables	xiii
List of Figures	xv
Introduction	1
Development of Duodenal Morphology	1
Differentiation of the Duodenal Epithelium	2
Development of the Brush Border	3
Histochemical Localization of Alkaline Phosphatase	4
Development of Duodenal Alkaline Phosphatase in the Chicken Embryo	5
Development of Alkaline Phosphatase in the Mouse	6
Function of Duodenal Alkaline Phosphatase	7
Effects of Corticoids on Functional Differentiation	8
Effects of Corticoids on Duodenal Development *In Vitro*	9
Duodenal Development in Adeneohypophyseoprivic Chicken Embryos	10
Effects of Hypophysectomy on Duodenal Alkaline Phosphatase in Rats	11
Effects of Thyroxine on Duodenal Development in Chicken Embryos	12
Thyroid Hormone Levels in Chicken Embryos	14
T_3 and T_4 in Birds	15
Effects of Thyroxine on Other Aspects of Intestinal Development	16
Direct Effects of Thyroxine on Duodenal Development	17

Materials and Methods 19

I. Experiments Performed *In Vivo* 19

II. Experiments Performed *In Vitro* 25

III. Biochemical Analysis 29

1. Preparation 29

2. Protein Determination 30

3. Alkaline Phosphatase Determination 33

4. Statistical Analysis 36

IV. Morphological Analysis 37

Results 40

I. Biochemical Analysis 40

A. Results of Experiments Performed *In Vivo* 40

1. General 40

2. Duodenal Protein Content 41

a. Stage 41

b. Time Pool 47

3. Alkaline Phosphatase Specific Activity 50

a. Stage 50

b. Time Pool 55

4. Weights 55

5. Stages of Development 57

6. Yolk Sac Retraction 60

7. Mortality 62

B. Results of Experiments Performed *In Vitro* 64

1. General 64

2. Total Protein Content of Duodenal Quarters 65

3. Alkaline Phosphatase Specific Activity 69

II. Morphological Analysis 72

A. Introduction 72

B. Experiments Performed *In Vivo* 72

1. Light Microscopy 73

2. Electron Microscopy 74

a. Absorptive Cells 74

Form 74

Brush Border 75

Terminal Web Region 76

Cytoplasm 76

Mitochondria 77

Golgi Apparatus 78

Lysosomes and Related Bodies 78

Glycogen 79

Lipid Droplets 80

Nucleus 80

Microfilaments and Microtubules 81

b. Goblet Cells 81

C. Experiments Performed *In Vitro* 82

1. Light Microscopy 82

2. Electron Microscopy 83

a. Absorptive Cells 83

Absorptive Cells Showing Normal Appearance 84

Absorptive Cells Showing Condensed Appearance 85

Brush Border - Normal and Condensed Absorptive Cells 86

b. Goblet Cells 87

Discussion 107

Experiments Performed *In Vivo* 107

Treatment Time 107

Stage 45 107

Effects of Thyroxine Treatment on Stage 108

Effects of Thyroxine on Yolk Sac Retraction 109

Mortality 109

Variation in Alkaline Phosphatase Specific Activities 111

Effects of Thyroxine Treatment on Duodenal Alkaline Phosphatase 113

Effects of T_4 Treatment on Duodenal Protein Content 117

Factors Contributing to Structural Variation in the Duodenal Epithelium 119

Effects of Thyroxine Treatment on the Duodenal Epithelium 121

Golgi Activity 122

Lysosomal Activity 123

Endocytosis 124

Brush Border 126

Glycogen 127

Lipid Droplets 129

Ribosomes and Endoplasmic Reticulum 131

Experiments Performed *In Vitro* 134

Culture Technique 134

Morphology of Duodenal Explants Cultured *In Vitro* 135

Brush Border of Cultured Epithelium 138

Goblet Cells 140

Biochemical Results 142

Effects of D-T_4 on Duodenal Explants 145

Effects of Culture Medium on Biochemical Results 146

Conclusions 149

Summary 151

Bibliography 154

Appendices

I. Materials and Methods: Sample Calculation of Total Duodenal Protein 175

II. Materials and Methods: Sample Calculation of Alkaline Phosphatase Specific Activity 177

III. Materials and Methods: Alkaline Phosphatase Determination Using Enzyme Commercially Prepared From *E. Coli* 180

IV. Results: Determination of Variation Due to Biochemical Methods 182

V. Materials and Methods: Duncan's New Multiple Range Comparison 189

VI. Results: Duodenal Fresh Weight Following Experiments Performed *In Vivo* 194

1. Fresh Weight 194

a. Stage 194

b. Time Pool 196

2. Dry Weight 198

a. Stage 198

b. Time Pool 200

3. Comparison of Fresh and Dry Weights 202

VII. Results: Body Weight Following Experiments Performed *In Vivo* 204

1. Stage 204

2. Time Pool 206

VIII. Results: Determinations of Alkaline Phosphatase Specific Activity, Protein Content, and Weight of Normal Duodenal Quarters 208

1. Protein Content 208

2. Alkaline Phosphatase Specific Activity 211

3. Fresh Weight 212

4. Dry Weight 213

5. Relationship Between Fresh and Dry Weight 214

IX. Results: Duodena Cultured 24 Hours *In Vitro* 216

1. Total Protein Content 216

2. Alkaline Phosphatase Specific Activity 218

3. Fresh Weight 219

4. Dry Weight 219

X. Results: Duodenal Quarters Cultured 48 Hours *In Vitro* 221

1. Stage 42 221

a. Fresh Weight 221

b. Dry Weight 225

2. Stage 43 226

a. Total Protein Content (Quarters) 226

b. Alkaline Phosphatase Specific Activity 226

c. Fresh Weight 228

d. Dry Weight 229

XI. Results: Duodenal Quarters Cultured 72 Hours *In Vitro* 230

1. Protein Content 230

2. Alkaline Phosphatase Specific Activity 231

3. Fresh Weight 231

4. Dry Weight 232

XII. Results: Determinations of Protein Content and Alkaline Phosphatase Specific Activity of Culture Media 234

List of Tables

		Page
1.	Experimental Design: Experiments Performed *In Vivo*	23
2.	Experimental Design: Experiments Performed *In Vitro*	28
3.	Duodenal Alkaline Phosphatase Sp. Act., Protein Content, Fresh and Dry Weight, and Body Weight of Normal Stage 41 to 45 Embryos.	42
4.	Total Protein Content of Duodenal Homogenates - Staged Results	45
5.	Total Protein Content of Duodenal Homogenates - Pooled According to Time	48
6.	Alkaline Phosphatase Sp. Act. of Duodenal Homogenates - Staged Results	51
7.	Alkaline Phosphatase Sp. Act. of Duodenal Homogenates - Pooled According to Time	53
8.	Distribution of Developmental Stages	58
9.	Yolk Sac Retraction	61
10.	Mortality	63
11.	Protein Content and Alkaline Phosphatase Specific Activity of Stage 42 Duodenal Quarters Cultured 48 Hours *In Vitro*	67
A-1.	Summary of Sample Separation and Dilutions For Determination of Variation Due to Biochemical Methods I	186
A-2.	Variation in Total Duodenal Protein and Alkaline Phosphatase Specific Activity Due to Biochemical Methods I	187
A-3.	Variation in Total Duodenal Protein and Alkaline Phosphatase Specific Activity Due to Biochemical Methods II	188
A-4.	Fresh Duodenal Weight - Staged Results	195
A-5.	Fresh Duodenal Weight - Results Pooled According to Time	197
A-6.	Dry Duodenal Weight - Staged Results	199

A-7. Dry Duodenal Weight - Results Pooled According to Time 201

A-8. Relationship Between Fresh and Dry Duodenal Weight 203

A-9. Body Weight - Staged Results 205

A-10. Body Weight - Results Pooled According to Time 207

A-11. Total Protein Content, Alkaline Phosphatase Specific Activity, and Fresh and Dry Weights of Duodenal Quarters From Normal Embryos.

I. Stages 42 and 43 209

II. Stages 44 and 45 210

A-12. Total Protein Content, Alkaline Phosphatase Specific Activity, Fresh and Dry Weights of Duodenal Explants Cultured 24 Hours *In Vitro* 217

A-13. Fresh and Dry Weights of Stage 42 Duodenal Quarters Cultured 48 Hours *In Vitro* 222

A-14. Results of Duncan's New Multiple Range Analysis Including Weight Data From Stage 42, 48 Hour Cultures With and Without BSA in the Same Comparison 224

A-15. Protein Content, Alkaline Phosphatase Specific Activity, and Fresh and Dry Weights of Stage 43 Duodenal Quarters Cultured 48 Hours *In Vitro* 227

A-16. Protein Content, Alkaline Phosphatase Specific Activity, and Fresh and Dry Weights of Stage 42 and 43 Duodenal Quarters Cultured 72 Hours *In Vitro* 233

A-17. Protein Content and Alkaline Phosphatase Specific Activity of Culture Media 236

List of Figures

		Page
1.	Total Protein Content of Duodenal Homogenates - Stage	46
2.	Total Protein Content of Duodenal Homogenates - Time Pool	49
3.	Alkaline Phosphatase Specific Activity - Stage	54
4.	Alkaline Phosphatase Specific Activity - Time Pool	56
5.	Distribution of Developmental Stages	59
6.	Total Protein Content of Stage 42 Duodenal Quarters Cultured 48 Hours *In Vitro*	68
7.	Alkaline Phosphatase Specific Activity of Stage 42 Duodenal Quarters Cultured 48 Hours *In Vitro*	70
8-22.	Light micrographs of duodenal epithelium of 42 to 45 embryos from experiments performed *in vivo*	89
23-38.	Electron micrographs of epithelial cells of stage 42 to 45 embryos from experiments performed *in vivo*	90-98
23.	Stage 42 Normal Absorptive Cells	90
23a.	Basal Lamina, microfilaments and secondary lysosome	90
24.	Apical cytoplasm of stage 42 normal absorptive cell	91
24a.	Dense body	91
25.	Apical cytoplasm of stage 42 normal absorptive cell	92
25a.	Multivesicular body	92
26.	Stage 43 normal absorptive cells	92
26a.	Glycogen	92
27.	Stage 43 D-T_4 treated (24 hr) absorptive cells	93
28.	Stage 43 L-T_4 treated (24 hr) absorptive cells	93

29. Stage 44 normal absorptive cells 94

30. Stage 44 D-T_4 treated (48 hr) absorptive and pre-goblet cells 94

31. Stage 44 L-T_4 treated (48 hr) absorptive cells 95

32. Stage 45 normal absorptive cells 95

32a. Area adjacent to lipid droplet 95

33. Stage 45 D-T_4 treated (72 hr) absorptive cells 96

34. Stage 45 L-T_4 treated (72 hr) absorptive cells 96

35. Stage 45 L-T_4 treated (48 hr) absorptive cells 97

36. Stage 42 pre-goblet cell 97

37. Stage 42 brush border 98

38. Stage 45 brush border 98

39-46. Light micrographs of stage 42 duodena following 48 hours of culture *in vitro* 98-100

47-62a. Electron micrographs of stage 42 duodenal epithelium following 48 hr of culture *in vitro* 101-106

47. Absorptive cells cultured in normal medium 101

48. Condensed absorptive cells cultured in normal medium containing BSA 101

49-49a. Absorptive cells cultured with D-T_4 and BSA 102

50. Condensed absorptive cells cultured with 14 ng L-T_4/ml of medium 103

51. Moderately condensed absorptive cells cultured with 14 ng L-T_4/ml of medium and BSA 103

52. Absorptive cell cultured with 14 ng L-T_4/100 ml of medium 104

53. Moderately condensed absorptive cells cultured with 14 ng L-T_4/100 ml of medium and BSA 104

54-61. Microvilli following 48 hr of culture *in vitro* 105

62-62a. Goblet cell following 48 hr of culture *in vitro* 106

INTRODUCTION

Intestinal development in chicken embryos has been a subject of investigation since the early 1900's when Hilton (1902) studied the form and development of intestinal folds and villi. Present day investigations are still concerned with intestinal morphogenesis, as well as with the functional differentiation of the absorptive epithelium. Involved in functional differentiation are changes in the structure and enzymes of the epithelial cells that are believed to be in preparation for the post-hatching onset of intestinal function.

In chicken embryo duodenum the most studied enzyme with respect to this problem is alkaline phosphatase, (orthophosphoric monoester phosphohydrolase, E.C. 3.1.3.1). The present study investigates the role of thyroxine (T_4) in the functional differentiation of the duodenum, using alkaline phosphatase and cellular structure as indicators of development.

Development of Duodenal Morphology

The early development of the intestine and its derivatives in the chicken embryo has been reviewed by Rudnick (1952) and Deren (1968). The digestive tube originates from the endodermal and mesoblastic mesenchymal

layers of the splanchnopleure. The endodermal layer gives rise to the absorptive and glandular epithelium, while the muscular, vascular, and connective tissue components are derived from the mesoblastic mesenchymal layer

Hilton (1902) described the patterns of folding and villus formation which give rise to the mature intestinal topography in the chicken embryo, and his observations have been confirmed by several recent investigators (Coulombre and Coulombre, 1958;Hinni and Watterson, 1963;Stocum, 1966;Grey, 1972;Burgess, 1975, 1976; Tsai and Overton, 1976;Lim and Low, 1977). By 16 days of development, the folds which formed in the intestinal lining during the second week of development are becoming villi by unequal growth and separation from the tips downward, and new villi have appeared between the folds (Hilton, 1902). Subsequently, the villi increase in length but their numbers remain unchanged (Clarke, 1967).

Differentiation of the Duodenal Epithelium

By the tenth day of development, the chicken embryo's duodenum is lined by a simple epithelium composed of cuboidal cells;these become increasingly columnar in form as hatching (c. day 21) approaches and after it has occurred (Argesanu and May, 1938;Van Alten and Fennell, 1957;Hinni and Watterson, 1963; Penttilä and Gripenberg, 1969;Bellware and Betz, 1970;Hart and Betz, 1972a). As The epithelial cells become increasingly columnar, their apical cytoplasm enlarges and their nuclei assume basal

locations.

Accompanying these changes are changes in ultrastructure. The endoplasmic reticulum, Golgi apparatus, and mitochondria become increasingly prominent while free cytoplasmic ribosomes decrease (Dobbins et al., 1967; Penttilä and Gripenberg, 1969). Paralleling the decrease in ribosomes is a decrease in stainable cytoplasmic RNA visible using light microscopy (Moog, 1961b). During the days preceding and following hatching prominent lipid droplets in the cytoplasm of the epithelial cells disappear (Holman, 1969) and goblet cells increase in number (Hinni and Watterson, 1963; Bellware and Betz, 1970; Hart and Betz, 1972a).

The ultrastructure of the developing intestinal epithelium has been studied in several other vertebrates including: amphibians (Bonneville, 1963; Bonneville and Weinstock, 1970) rabbits (Deren et al., 1965; Deren, 1968), rats (Dunn, 1967), guinea pigs (Merrill et al., 1967), humans (Moxey and Trier, 1975), and mice (Calvert and Ménard, 1978).

Development of the Brush Border

As the epithelial cells become increasingly columnar, their luminal brush border is differentiating. Overton and Shoup (1964) reported the following steps in the development of the brush border in chicken embryos:

from day 11 to 16 the microvillous core filaments and rootlets appear and the microvilli come to form 90° angles with the apical surface, without increasing in height; from days 16 to 19 the microvilli increase moderately and thereafter rapidly in height until day 22. Several investigators have confirmed these observations (Hayes, 1976b; Penttilä and Gripenberg, 1969; Ono, 1973).

Histochemical Localization of Alkaline Phosphatase

Gombri (1941), using light microscope histochemistry, found that the brush border of the intestinal epithelium is a site of intense alkaline phosphatase activity. Using electron microscopic histochemistry, its location has been determined to be the outer portion of the microvillar membrane, coinciding with the portions of the glycocalyx adjacent to the membrane (Hugon and Borgers, 1966, 1969a,b; Ito, 1969; Ono, 1973). Fractionation studies have confirmed this location (Holt and Miller, 1962; Overton *et al.*, 1965), and have suggested that the enzyme is attached to the membrane by hydrophobic anchors (Louvard *et al.*, 1975). A recent review of the differentiation of the brush border membranes has been made by Michell *et al.*, (1976).

Development of Duodenal Alkaline Phosphatase in the Chicken Embryo

The large body of knowledge concerning the functional differentiation of alkaline phosphatase in the duodena of chicken embryos and post-natal mammals results mainly from the work of Moog and her colleagues. She has reviewed this subject several times (Moog, 1952, 1958, 1962, 1965a; Moog *et al.*, 1969).

During the second week of development, the alkaline phosphatase activity of whole chicken embryo homogenates was found to increase and the phosphatase activity of the intestine was much greater than that of several other tissues (Moog, 1946). Subsequently, Moog (1950) found that duodenal alkaline phosphatase increased slowly from 9 to 18 days of development, then rapidly until hatching. The level of enzyme then declined until the end of the first week of embryonic development and remained stable at that level (presumed the adult level) for 7 weeks (ibid.). If newly hatched embryos were starved for 48 to 96 hours they accumulated three times the amount of phosphatase found in fed chicks, and a delayed increase could be induced if chicks were fed, then starved (ibid.).

The increase in alkaline phosphatase could also be demonstrated histochemically. Moog (1950) found the cytoplasm of the epithelial cells to be weakly reactive at 16 days, the brush border reactive at 18 days, and the

Golgi region reactive at hatching. By 20 days the brush border was strongly reactive on the upper two thirds of the villi, and by hatching this extended into the crypts (ibid.). Overton and Shoup (1964) noted that the development of the brush border paralleled the increase in alkaline phosphatase demonstrated biochemically and histochemically by Moog (1950).

Subsequently, Hancox and Hyslop (1953) found the brush border to be positive for alkaline phosphatase as early as 14 days. More recently, using electron microscope histochemistry, Ono (1973) found that some patchy reaction product indicative of alkaline phosphatase activity was present on the first microvillar surface projections of eight day embryos.

Development of Alkaline Phosphatase in the Mouse

In the developing mouse duodenum a biphasic pattern of alkaline phosphatase increase was found (Moog, 1951). The first increase occurred as in the chicken embryo during the days preceding birth. A second increase then occurred immediately preceding weaning, prior to the mouse's first encounter with solid food. Starving accelerated the second increase without raising phosphatase above the normal level (ibid.). Thus increasing alkaline phosphatase activity prior to the onset (or change) of function seemed to be immediately related to this event in both chickens and mice.

As duodenal alkaline phosphatase accumulates in chickens and mice, it exhibits changes in substrate preference which have been the subject of several studies by Moog and colleagues (Kato, 1959; Moog, 1961a, 1962, 1964, 1965b, 1966; Moog and Grey, 1966, 1967, 1968; Grey and Moog, 1966; Etzler and Moog, 1968; Moog *et al.*, 1969; Yeh and Moog, 1973). The development of alkaline phosphatase isozymes in chicken embryo duodenum has also been studied (Chang and Moog, 1975a,b).

Function of Duodenal Alkaline Phosphatase

The function of duodenal alkaline phosphatase is as yet unclear. The ability of duodena to accumulate glucose *in vitro* develops simultaneously with normal or induced increases in alkaline phosphatase (Betz and Mallon, 1970), and active sugar transport begins around hatching (Bogner and Haines, 1964). However, alkaline phosphatase probably does not participate directly in sugar absorption (Crane and Krane, 1956).

It has also been implicated to function in fat absorption although a direct involvement has not been demonstrated (see review by Kaplan, 1972). Recently it has been suggested to function in thiamine absorption (Matsuda *et al.*, 1974). In chicken embryos phosphate absorption rises parallel to increased phosphatase activity, suggesting a function in this process (Moog and Glazier, 1972).

Thus far the function of duodenal alkaline phosphosphatase has been most studied with respect to its possible participation in vitamin D-induced intestinal calcium absorption (Martin et al., 1969; Haussler et al., 1970; Holdsworth, 1970; Melancon and DeLuca, 1970; Norman et al., 1970; Russel et al., 1972; Krawitt et al., 1973). Considerable controversy exists as to its involvement however, and to whether it is involved with or identical to vitamin D-induced calcium-dependent ATPase. In duodenal explants from 20 day chicken embryos, vitamin D_3 and its metabolites can induce increased calcium absorption, accompanied by increased alkaline phosphatase, although the concentrations which stimulate calcium absorption do not always stimulate phosphatase (Corradino, 1973).

Effects of Corticoids on Functional Differentiation

Early in the investigation of functional differentiation it became apparent that hormones were involved in its control. Adrenalectomy of suckling mice inhibited the second phase of alkaline phosphatase increase, and corticoid administration could cause the increase to occur early in intact mice (Moog, 1953). The latter response took 24 to 48 hours to occur, depending on how far ahead of the normal increase treatment had been, and the response could only occur during a limited period of development (ibid.).

In chicken embryos hydroxy-cortisone acetate and cortisone acetate were both found to elicit a precocious increase in duodenal alkaline phosphatase (Moog and Richardson, 1955). The period of responsiveness to corticoids was found to be limited from 10 to 18 days. At 16 days treatment produced a strong response within 48 hours and after 18 days responsiveness declined rapidly. Not only was phosphatase elevated by this treatment, but the entire histological development of the epithelial cells was accelerated as well (ibid.). Glycogen, which appeared in increasing quantities in the epithelial cells then disappeared rapidly, was deposited more quickly and depleted sooner in embryos treated with corticoids (Moog and Richardson, 1955; Moog and Thomas, 1957).

Effects of Corticoids on Duodenal Development *In Vitro*

The duodenum was also found to respond to corticoids when cultured *in vitro*. Duodena explanted from 16 day chicken embryos showed increased alkaline phosphatase after 24 to 48 hours of culture with hydrocortisone (Moog and Nehari, 1954; Moog and Kirsch, 1955). In these studies phosphatase accumulation and histological development were found to be independent. Hayes (1965b) found that cortisone could stimulate increased alkaline phosphatase in explants from embryos as young as 13 days, and that increasing the hormone concentration increased

the response in explants from 13 to 19 day embryos. Cortisone also promoted villar morphogenesis and increase in villar height (Hayes, 1965a). Pedernera (1970) has used this capacity to increase villar height in explanted chicken embryo duodenum as a bioassay for corticosteroids.

Duodenal Development in Adenohypophyseoprivic Chicken Embryos

Chicken embryos cannot be adrenalectomized, however the source of adrenocorticotrophin (ACTH) and therefore its stimulation of the adrenals can be removed by partial decapitation, which renders the embryos functionally adenohypophyseoprivic and is equivalent to hypophysectomy (see Betz, 1971, 1975). In partially decapitated chicken embryos, differentiation of the duodenal epithelium fails to proceed beyond the level normally attained at 17.5 days of development, the first effects appearing at day 14 (Hinni and Watterson, 1963; Bellware and Betz, 1970; Hart and Betz, 1972a). In these embryos alkaline phosphatase fails to increase, morphogenesis is slowed, absorptive cell differentiation ceases, and goblet cells fail to increase in number (Ibid.).

Normal duodenal development was restored by grafting cephalic or mid-regions of pars distalis onto the chorioallantoic membranes of partially decapitated embryos (Bellware and Betz, 1970; Hart and Betz, 1972a,b). Both ACTH and thyroid stimulating hormone (TSH) activities are

associated with these regions (Brasch and Betz, 1971).

Administration of thyroxine (0.2 μg on day 15 and 0.4 μg on days 16, 17, and 18) and cortisone acetate (1.25 mg on day 17.5) together did not restore normal development in partially decapitated embryos (Hinni and Watterson, 1963). Subsequently duodenal phosphatase in such embryos was found to be elevated at day 16.5 following daily injections from day 13.5 of 1.5 μg T_4, alone or in combination with a single treatment of hydrocortisone, (0.25 mg) on day 14.5 (Betz and Mallon, 1970; Mallon, 1972). However the effects of such treatments were usually toxic in partially decapitated embryos. The same treatment schedule and dosages were also found to elevate duodenal phosphatase at day 16.5 in intact embryos (ibid.).

Effects of Hypophysectomy on Duodenal Alkaline Phosphatase in Rats

In young rats which have been hypophysectomized duodenal alkaline phosphatase fails to increase above the neonatal level (Yeh and Moog, 1973, 1975a). Their villi are short and the mitotic rate subnormal (Yeh, 1977), and the cells exhibit less endocytosis, rough endoplasmic reticulum, and Golgi activity than normal (Yeh and Moog, 1975a, 1977b). Thyroxine treatment restored phosphatase levels and ultrastructure to normal, while cortisone acetate did not restore phosphatase and produced abnormal Golgi complexes (Yeh and Moog, 1975b). Thyroidectomy of

young rats produced effects similar to those produced by hypophysectomy, and similarly thyroxine was more effective than cortisone in restoring alkaline phosphatase (Yeh and Moog, 1973, 1977a). Both operations also reduced jejunal maltase and sucrase (invertase) and corticoids repaired their levels while thyroxine did not (Yeh and Moog, 1973, 1975b, 1977a). Recently cortisone has been found to restore reduced glycoprotein synthesis, as observed by ^{3}H-galactose migration in hypophysectomized rats (Yeh and Moog, 1977b).

Effects of Thyroxine on Duodenal Development in Chicken Embryos

Moog (1961b) found that administration of the goitrogen thiourea to chicken embryos on day 11 inhibited the normal pre-hatching increase in duodenal alkaline phosphatase. The embryos failed to hatch and remained alive in their shells up to one week longer than normal. During this period cellular differentiation proceeded slowly (although more glycogen accumulated than normal) and phosphatase increased slowly but never exceeded more than one tenth of the normal level. Treatment of such embryos with L-thyroxine (L-T_4) on days 13 and 15 (total dosage 3.0 mg) restored phosphatase and other aspects of differentiation to normal by day 18 (ibid.).

The effects of thiourea treatment could also be restored by the administration of L-T_4 with cortisone

acetate, but the latter hormone alone only raised phosphatase slightly and did not restore it to normal (ibid.). Adrenal growth and differentiation were not affected by thiourea treatment (ibid.).

Using the same treatment schedule and dosages as for thiourea-treated embryos, Moog (1961b) also treated normal embryos, and found that a slight (insignificant) increase in duodenal alkaline phosphatase followed. She concluded that thyroxine did not influence phosphatase accumulation in otherwise normal embryos, but that it was probably 'permissive' to the stimulatory role of corticoids in intestinal development, acting at the level of the responding tissue. Hulan and Betz (1973) found that duodenal phosphatase at 19 days was significantly elevated following metopirone treatment, believed to block synthesis of adrenal corticoid hormones. However, Wishart et al., (1977) have indicated that metopirone may actually increase glucocorticoid output. Stocum (1966) found that cortisone acetate initially accelerated morphogenesis, but appeared inhibitory after 19 days. He also found that thiourea treatment produced morphological effects similar to those produced by partial decapitation, although the latter were more profound, and suggested that normal T_4 levels must be maintained during duodenal development.

Thyroid Hormone Levels in Chicken Embryos

In chicken embryos made adenohypopyseoprivic ('hypophysectomized') by partial decapitation, the level of total circulating T_4 fails to rise above the level found in normal 11 day embryos (Daugeras-Bernard et al., 1969; Thommes et al., 1977). In normal embryos T_4 continues to rise, peaking at day 16.5 (Thommes et al., 1977) or 17 (Davison, 1976). Following this peak there is a slight decline, then a second peak immediately following hatching (Davison, 1976). Thomes and Hylka (1977) found plasma T_4 to rise to day 19.5 then decrease significantly by day 21. Free circulating T_4 levels have been found to correlate well with the peripheral tissue effects of thyroid hormones in warm-blooded vertebrates (Refetoff et al., 1970), and free T_4 peaks at day 17 (Davison, 1976).

The plasma concentration of 3,3',5-triiodothyronine (T_3) also increases in the developing chicken embryo, gradually from days 11.5 to 15.5, then sharply until just after hatching when T_3 begins to decline (King et al., 1977; Thommes and Hylka, 1977). The plasma concentration of 3,3',5'-triiodothyronine (reverse T_3 or rT_3) also increases gradually to day 19.5 then begins to decrease (Thommes and Hylka, 1977). As well, plasma corticosterone increases and peaks shortly before hatching (Wise and Frye, 1973; Siegel and Gould, 1977).

T_3 and T_4 in Birds

In chickens, turkeys, and ducks, T_4 has been found to constitute approximately 60% and T_3 40% of the circulating thyroid hormone (Wentworth and Mellen, 1961). The potency of the two hormones has been found to be equal with respect to several criteria in thiouracil treated chickens, however T_4 was found to be the most potent in antigoiterogenic activity (Newcomer, 1957). Shellabarger (1955) found both hormones to be equipotent in goiter prevention in chicks, and T_4 has been found to be more effective in reducing TSH secretion in fowl (Mellen and Wentworth, 1959). In young chicks T_3 may be more important in controlling metabolic rate (Bobek et al., 1977). The half-lives of T_3 and T_4 in avian plasma are approximately equal (Tata and Shellabarger, 1959; Heninger and Newcomer, 1964). In mammals T_3 is much more potent than T_4; this difference between birds and mammals is as yet unexplained, but is apparently not due to the binding proteins present in the serum of the two groups (Tritsch and Tritsch, 1965). Reineke and Turner (1945) found that L-T_4 was twice as active as D,L-T_4 in chickens, and therefore concluded that the D-compound has little or no activity. Diurnal rhythms of thyroid function found in chicks (Newcomer, 1974) have not been reported in embryos.

Effects of Thyroxine on Other Aspects of Intestinal Development

An increasing amount of evidence is accumulating that thyroxine is active in many aspects of intestinal maturation. In suckling rats T_4 treatment causes: precocious closure or cessation of macromolecular uptake by the intestine (Chan et al., 1973); precocious increase in α-disaccharidases (Koldovský et al., 1974); and precocious decrease in ileal acid hydrolase activity (ibid.). Thyroidectomy of suckling rats delays the usual increases in jejunal sucrase and maltase (Yeh and Moog, 1973, 1977a), as well as decreases in lysosomal hydrolases (Koldovský et al., 1975) and jejunal lactase (Yeh and Moog, 1974). In adult rats, T_4 treatment will produce a decrease in jejunal lactase (Celano et al., 1977). Blanes et al. (1977) found decreased villus height and mucosal thickness in newborn induced hypothyroid rats.

There is also evidence accumulating to implicate interaction of T_4 and corticoids in these systems. Malinowska et al. (1974) found that plasma cortocoid concentrations increased in T_4 treated neonatal rats, suggesting that T_4 induced closure might be via corticoids. Thyroxine has also been suggested to be permissive in jejunal sucrase ontogeny in suckling rats, with timing possibly dependent on circulating T_4 levels, but corticoids being the primary inducers (Henning, 19[illegible]

Kaltenbach et al. (1978) found that premature amphibian metamorphosis induced by T_4 was accompanied by

precocious changes in intestinal alkaline phosphatase, and that changes in alkaline phosphatase normally occur during metamorphosis.

Direct Effects of Thyroxine on Duodenal Development

Recently evidence for direct action of thyroxine on the chicken embryo duodenum has been found using organ culture. In duodena explanted from 14 day embryos and cultured 48 hour *in vitro* in the presence of T_4, goblet cell numbers are increased compared to cultures without hydrocortisone (Black and Moog, 1977). In cultures with hydrocortisone this increase does not occur (ibid.), contrary to the increase in goblet cell number induced precociously by hydrocortisone treatment of chicken embryos (Moog and Richardson, 1955).

In another recently published study, Black and Moog (1978) found that T_4 produced increased alkaline phosphatase within 48 hours in duodenal explants from 14 day embryos. Cortisone elevated phosphatase only slightly, but produced precocious changes in the rate of substrate hydrolysis. Maltase rose at the same rate as *in vivo* with either T_4 or cortisone but did not rise in unsupplemented medium (ibid.). Large amounts of both enzymes were lost to the medium, paralleling the increases found in the explants. Previously Struthers (1970) had found that T_4 inhibited alkaline phosphatase increase in explanted duodenum, however, the hormone concentration which he used was much greater

than those used by Black and Moog (1978).

Therefore thyroxine is apparently involved in several aspects of intestinal development in different species. The present study was attempted in order to re-investigate the role of T_4 in the stimulation of the normal pre-hatching increase in alkaline phosphatase in chicken embryos. Thyroxine was administered to normal embryos in a larger dosage and closer to the normal pre-hatching phosphatase increase than in previous studies (Moog, 1961b; Betz and Mallon, 1970; Mallon, 1972). Subsequent changes in duodenal alkaline phosphatase, protein content, and cellular structure were then monitored. A direct effect of T_4 on the duodenum was also tested by the culture of duodenal explants in synthetic medium. Organ culture additionally confirmed that duodena of a known stage were treated. In addition, the effects of D-T_4, used as a physiocochemical control, were tested *in vivo* and *in vitro*.

MATERIALS AND METHODS

I Experiments Performed *In Vivo*

Fertilized White Leghorn eggs (*Gallus gallus*), strain Hyline 934F, were obtained from a commercial supplier (Sterling McEwan, North Gower, Ontario). Prior to incubation the eggs were stored a maximum of one week at 20°C. The eggs were incubated to 38 ± 0.5°C and 60 ± 5% relative humidity (Coldstream forced draft incubator) in darkness on foam rubber nests (Betz, 1963). After 34 to 40 hours of incubation, eggs were windowed using a diamond dust impregnated cone driven by a dental motor mounted on a drill press (see Betz, 1975). Prior to windowing, each egg was swabbed with 70% ethyl alcohol and a hole made through the shell into the air space at the blunt end using a steel needle approximately 1 mm in diameter and 2-3 mm in length, mounted on a wooden handle. Circular windows (10 mm diam) were made by touching the cone to the shell while running the drill at high speed. The egg dust produced was then blown away from the window, and the shell piece and shell membranes removed using blunted forceps (no. 5). If the egg contents did not drop away from the window when the shell membrane was pierced, the egg was discarded. The windows were sealed

using Scotch ® Brand Magic Transparent Tape no. 810 (3M, 12.7 mm wide) which was burnished around the perimeter to promote secure adhesion. Eggs cracked during the windowing procedure were repaired with tape if possible, but badly damaged eggs were discarded. All successfully windowed eggs were kept at this time, even if no blastoderm was visible, to ensure that no fertile eggs would be discarded. Survival after windowing was improved by flipping the egg before drilling so that the blastoderm would be away from the site of the operation, and by removing each basket of eggs from the incubator only for the time required for windowing its eggs.

Three days following windowing, the eggs were candled and unfertilized eggs and those containing dead embryos were discarded. Swabbing of the tape with 70% alcohol rendered it transparent long enough to observe the state of the embryonic blood circulation (which was well developed by this time). During the following days the eggs were checked every 48 hr and dead embryos discarded to reduce the chance of infection. Throughout incubation the positions of the egg baskets were rotated in the incubator to standardize incubation conditions.

A control group of unwindowed eggs was maintained throughout the procedure and exposed to the same amount of handling as the windowed eggs. This group was

also candled and its position changed during incubation.

Treatments consisted of single injections of 5.7 μg L-T_4 (3,3',5,5'-tetraiodo-L-thyronine pentahydrate salt; Sigma, St. Louis Mo., lot number 103C-2150), or 5.7 μg D-T_4 (3,3'5,5'-tetraiodo-D-thyronine pentahydrate salt; Sigma, lot no. 91C-1170), equivalent to 5 μg[1] hormone in each case, in 0.1 ml saline vehicle. The optical purity of each compound was verified by the supplier to be greater than 99.99%, and the optical rotations of injection solutions were confirmed polorimetrically. Thyroxine was initially dissolved in alkalinized 0.9% saline (pH greater than 8.3) and the pH then lowered to 7.4 (final pH).

After 16 days of incubation (total) the windowed eggs were randomly grouped for treatment i.e. eggs from each basket were included in all groups. Four eggs were opened to determine that an average stage of 42 had been reached according to Hamburger and Hamilton's stages of normal development (1951). Injection of T_4 treatment and vehicle control solutions was performed after approximately 392 hours of incubation by applying 0.1 ml of solution from a hypodermic syringe onto the chorioallantoic membrane beneath the window, without abrading or puncturing the membrane. Treatment

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1 This dosage was recommended to Dr. T.W. Betz by Dr. R.C. Thommes, Dept. of Biological Sciences, De Paul University, Chicago, Ill. in a personal communication.

was always performed at 1100 Hrs Eastern Standard Time, and all windowed embryos were inspected and only healthy embryos used (i.e. embryos with bright red blood vessels and healthy chorioallantoic membranes, and showing body movement). Unwindowed eggs were candled to eliminate those containing dead embryos. Following treatment, the windowed eggs were re-sealed with tape and re-incubated. All eggs were left undisturbed until the times the embryos were to be killed.

The treatment groups were comprised of the following individuals:

1) Normal unwindowed eggs. These were the same age and received similar handling to the windowed groups.
2) Windowed eggs, uninjected.
3) Saline-injected, windowed, treated with 0.1 ml 0.9% sterile saline vehicle.
4) D-T_4 treated windowed, treated with 5.7 μg D-T_4 (pentahydrate salt) in 0.1 ml saline vehicle.
5) L-T_4 treated, windowed, treated with 5.7 μg L-T_4 (pentahydrate salt) in 0.1 ml saline vehicle. (Refer to Table 1.)

Following treatment the eggs were re-incubated for 24, 48, or 72 hours without disturbance. At each of these times, the egg shells were cut open using scissors and the embryos killed by decapitation (also using scissors). The yolk sac was cut away from the embryo's

Table 1

Experimental Design: Experiments Performed *In Vivo*

Group	Treatment	Number	Sample Size
Normal	Unwindowed, no treatment	144	93
Windowed	Windowed, no treatment	88	67
Saline	Windowed, treatment: 0.1 ml 0.9% saline vehicle	124	100
D-T_4	Windowed, treatment: 5.7 µg D-T_4 (pentahydrate salt) in 0.1 ml 0.9% saline vehicle	170	119
L-T_4	Windowed, treatment: 5.7 µg L-T_4 (pentahydrate salt) in 0.1 ml 0.9% saline vehicle	360	162

Number refers to the number of individuals in each group at the time of treatment. Sample size refers to the number of individuals surviving to treatment time and used to obtain data. For the mortalities at each sample time refer to Table 10.

abdomen and the abdominal wall cut open to expose the duodenal loop. In the earlier embryonic stages it was often covered by other portions of intestine, but in the later stages it usually bent over the other loops and lay partially exposed across them. The duodenal loop was quickly removed from each embryo by making a single cut through both arms 2 mm from the stomach. This procedure removed the entire duodenum from the pyloric region to the entrance of the common bile duct, and although approximate, was consistantly reproducible. Pancreas and mesentery were removed from the loop prior to light blotting and weighing to the nearest 0.1 mg (Cahn Millibalance 7500). Each duodenal loop was then either fast frozen using a dry ice-acetone bath, for later biochemical analysis, or fixed in half strength Karnovsky fixative (Karnovsky, 1965), prior to processing for microscopy as described in section IV (Materials and Methods). If a duodenal loop was physically damaged, i.e. torn or crushed during its removal or accompanying procedures, it was discarded.

All embryos were staged according to Hamburger and Hamilton's stages of normal development (Hamburger and Hamilton, 1951) using beak and toe length measurements. The embryos were weighed (head and body) to the nearest 0.1 g (Mettler balance). Relative amounts of yolk sac retraction were noted visually as was the presence of

pipping (breaking of the shell in a small area by means of the egg tooth, the first externally visible sign of hatching). Some of the embryos were fixed in 40% formaldehyde solution for future reference and the rest were discarded. The proportion of embryos with gross morphological defects (e.g. exposed brains, deformed limbs, extreme underdevelopment) was noted, however such embryos were discarded and their duodena were not used. The distributions of developmental stages in the various treatment groups were compared using tables of binomial confidence limits (in Steel and Torrie, 1960).

II Experiments Performed *In Vitro*

A second set of experiments involved organ culture of embryonic duodena (after Corradino, 1973) in the presence of T_4-containing media. Preliminary to culture, eggs of the same strain were incubated as described in section I for approximately 392 hours. These were then candled and swabbed with 70% alcohol. Under aseptic conditions (EdgeGARD Hood, Baker Co. Inc. Sanford, Maine) the eggs were opened and the embryos killed by decapitation and staged. The duodena of stage 42 embryos were removed and cleaned as in the experiments *in vivo*, then immersed in sterile 0.9%

saline on glass slides. Using a sharp single-edged razor blade each duodenum was cut into four segments of approximately equal length i.e. transverse cuts were made at the mid-points of each arm and of the bend in the loop. These segments were designated by the letters A through D from the proximal to distal segment.

Each duodenal quarter was lightly blotted then weighed to the nearest 0.1 mg (see Appendices) and re-immersed in sterile saline. Using a sharp blade each quarter was slit open lengthwise then cultured mucosal side up on stainless steel grids (60 mesh) in plastic petri dishes (100 x 75 mm) (Corradino, 1973). The grids were cut from sheets of stainless steel mesh to a size suitable for the dishes used (approximately 5.0 x 7.0 mm) and the short ends bent under (0.5 mm) to form supporting edges at right angles to the grid surface. New grids were treated in the following manner prior to sterilization (autoclave): boil in 10% $NaCO_3$ for 15 min., rinse in H_2O, soak in pure nitric acid overnight, rinse 6 to 8 times in H_2O, wash in Decon 75 ® solution, rinse in H_2O, then 70% ethanol, each 6 to 8 times.

McCoy's 5A culture media (modified) without fetal calf serum or antibiotics, with L-glutamine, and with sodium bicarbonate added (Cat. No. H-15, Grand Island Biological Co., Grand Island, N.Y.) was brought to the surface of the grids so as to wet the duodenal surface

without floating the pieces (approximately 30 ml/dish). Media were made fresh for all experiments and sterilized by vacuum filtration (Nalgene ® Filter Units 0.2 μ pore size, Nalge Sybron Corp., Rochester, N.Y.). Thyroxine (L-T_4 and D-T_4 dissolved separately as previously described) was added to the media at the time of filtration (0.1 ml saline vehicle to 99.0 ml media) to produce hormone concentrations of 14 ng/ml or 14 ng/100 ml. These levels were based on the circulating embryonic free T_4 level as determined by Davison (1976). (See Table 2 for summary of experimental design.) For controls, 1.0 ml of 0.9% saline was added to untreated media. In a second set of experiments, bovine serum albumin (Fraction V, Sigma) was added to the media to a concentration of 0.2% (200 mg/100 ml). The pH of all media was adjusted to 7.4 prior to culture. (McCoy's 5A contains phenol red as a pH indicator.)

The duodenal quarters were randomly assigned to the different treatment dishes i.e. different segments of the same duodenum were placed in culture dishes containing different treatment media. The cultures were incubated for 48 hours at 38 ± 0.5°C and approximately 90% relative humidity in a 5% CO_2 in air atmosphere (National 3321 Water Jacketed Incubator, Portland, Oregon). The media were not changed during the culture periods.

At the termination of culture, the pH of the different media was usually found to have dropped to about

Table 2 Experimental Design: Experiments Performed In Vitro

Group	Culture Medium
Normal (N)	McCoy's 5A + 0.9% saline
D	McCoy's 5A + D-T_4
L	McCoy's 5A + L-T_4
N + BSA	McCoy's 5A + 0.9% saline + bovine serum albumin (BSA)
D + BSA	McCoy's 5A + D-T_4 + BSA
L + BSA	McCoy's 5A + L-T_4 + BSA

All hormones (pentahydrate sodium salts) were dissolved in 0.9% saline and added to the culture media (1.0 ml saline + 99.0 ml medium) to concentrations of 14 ng/ml and 14 ng/100ml. Bovine serum albumin was added in a concentration of 200 mg/100 ml (0.2%).

7.2. If a larger change in pH had occurred, these segments were discarded. The quarters were removed from the grids, lightly blotted, weighed to the nearest 0.1 mg (fresh weight after culture) and either quick-frozen using a dry-ice-acetone bath for subsequent biochemical analysis, or fixed (Buckley, 1973) for subsequent microscopic processing. (Refer to following sections.)

For comparison, duodenal segments were removed from normal embryos at stages corresponding to those of the cultured material and subjected to biochemical analysis (see Appendix).

III Biochemical Analysis

1. Preparation

Following removal from the embryos or culture, duodena or duodenal quarters were blotted lightly, weighed (fresh weight or fresh weight after culture), placed in glass vials and quick-frozen in a dry ice-acetone bath. The frozen duodena were then lyophilized 48 hours to constant weight (Virtis Unitrap lyophilizer, Gardiner, N.Y.) and dry weights to the nearest 0.1 mg recorded. The samples were then stored at -20°C until analysis could be performed.

Each lyophilized duodenal sample was homogenized separately in a ground glass homogenizer containing 1.0 ml ice cold glass distilled water, using a ground glass pestle mounted on a Con-Torque stirrer (Eberbach Corp., Ann Arbor, Michigan). Homogenation was done at high speed for 15 min in an ice bath. Following this procedure, light microscopic examination revealed the presence of particulate material (approx. 3 μ or less in diameter) and some larger pieces of debris, none of which resembled intact cells. The homogenate was stored overnight (4°C) and the volume then brought to 2 ml with glass distilled water. Mixing of the homogenate was accomplished using a Vortex-Genie mixer and by drawing the material in and out of a Pasteur pipette several times. When the homogenate had been well mixed it was divided as follows: 0.5 ml for protein determination, 0.5 ml for alkaline phosphatase determination, and 1.0 ml frozen (-20°C) as reserve. The divisions were carried out in ice baths, and between steps the material was returned to the refrigerator (4°C).

2. Protein Determination

Prior to determination of total duodenal (or segment) protein by the Lowry (1951) procedure, the protein was separated by the following common biochemical methods. One ml of 10% (w/vol) trichloroacetic acid

(TCA) was added to the 0.5 ml homogenate. After vigorous mixing the homogenate-TCA mixture was boiled (100°C) for 15 min then centrifuged 10 min at 700 x g (2000 rpm, Sorvall GLC-1 centrifuge). The pellet was re-suspended in 1 ml 10% TCA, stored overnight (0°C), then spun 10 min at 700 x g. The final pellet was dissolved in 3 to 10 ml 0.5 N NaOH (3 to 14 hr @ 60°C) depending on the protein content of the sample.

Reagents used in protein determination, prepared according to Lowry (1951), were as follows:

Reagent A: 2% Na_2CO_3 in distilled water

Reagent B: 0.5% $CuSO_4 \cdot 5H_2O$ in 1% sodium tartrate

Reagent D: Reagents A and B freshly mixed, 50.0 ml A to 1.0 ml B

Reagent E: 1N phenol reagent prepared from 2N phenol reagent (Folin-Ciocalteau) by dilution with distilled water.

Reagent D is used for protein samples dissolved in base, while reagent C (not used here) is prepared from reagent A made as a basic solution mixed with B.

The procedures were carried out at room temperature in standard glass test tubes. To duplicate 1.0 ml protein samples (dissolved in 0.5 N NaOH) 5.0 ml reagent D were added with immediate vigorous mixing. The tubes were allowed to stand 10 min, then 0.5 ml freshly made reagent E was added to each tube with

immediate vigorous mixing. After standing 30 min for maximal colour development the samples were read at 560 nm (Hitachi Perkin-Elmer 139 spectrophotometer) against a reagent blank, prepared by adding the reagents to 1.0 ml 0.5 N NaOH according to the same schedule. From each sample of a duplicate pair two aliquots were read; these provided identical values. The two samples of a duplicate pair differed in optical density to a maximum of 5%.

For each day's analysis a new standard curve was prepared. From a stock solution of 250 μg bovine serum albumin (BSA, essentially fatty acid free, prepared from fraction V, Sigma)/ml 0.5 N NaOH, five duplicate 1.0 ml dilutions (50 to 250 μg) were made. These were treated in the same manner as the regular samples. From the resultant curve a slope conversion factor (p) was obtained. As the curve was linear in the range used, a single factor derived from the optical density at 100 μg protein was used. Total duodenal protein was then calculated using the following formula: Total protein (mg) = OD x p x df; where OD is the observed optical density (560 nm) and df the dilution factor. For a sample calculation refer to Appendix I.

As optical densities can be read more accurately in the lower range and the standard curve tends to level off at high optical density (Lowry, 1951), dilution factors

were adjusted according to the protein content of the samples as estimated in trial runs.

3. Alkaline Phospatase Determination

Duodenal alkaline phosphatase was determined by a modification for tissue homogenates (Manning et al., 1967) of Babson et al.'s (1966) technique. This technique uses phenolphthalein monophosphate as a substrate. Its hydrolysis at a rate linear to enzyme concentration and incubation time provides a chromogen in the form of free phenolphthalein for direct spectrophotometric determination. For every mole of phenolphthalein monophosphate hydrolyzed, one mole of phosphate and one mole of phenolphthalein are liberated.

From the original duodenal homogenate (2.0 ml), 0.5 ml was reserved for alkaline phosphatase analysis. The homogenate can be kept for up to a week at 4°C with no loss in alkaline phosphatase activity (Goldberg, 1971), however here analysis was usually done within three days of homogenizing. Just prior to analysis the homogenates were diluted to the desired concentrations with distilled water (dilution factors 20 to 200), and were kept at 4°C until needed.

In standard glass test tubes the substrate solution was prepared by adding 1 drop (0.04 ml) of

Phosphastrate ® Alkaline Reagent (General Diagnostics, Warner-Lambert Co., White Plains, N.J.) to 1.0 ml glass distilled water, then mixed using a Vortex-Genie. The substrate produced (2.5 mM phenolphthalein monophosphate in 0.3 M 2-amino-2-methyl-1-propanol) had a pH of 9.9. The tubes were placed in a 37.5 ± 0.5°C water bath to equilibrate (10 min). During this time the dilute homogenate samples were also placed in the bath to equilibrate.

The other reagent used was a colour stabilizer, 0.1 M phosphate buffer, pH 11.2, made by dissolving 9.3 g $Na_3PO_4 \cdot 12\ H_2O$ and 20.3 g $Na_2HPO_4\ 7H_2O$ in water which was brought to a volume of one liter. This was stored at 4°C and also allowed to equilibrate before use.

At timed intervals, 0.1 ml of sample was added to a substrate-containing tube, which was immediately mixed (using a Vortex-Genie) and returned to the 37.5°C water bath. Intervals of 30 seconds were found to be convenient, allowing adequate time for mixing and transfer (Eppendorf micro-pipettes) of the homogenate, mixing of the sample and substrate, and return of the reaction tube to the water bath. At exact intervals, following an incubation time of 10 to 60 min (usually 20), the reactions were terminated by the addition of 5.0 ml colour stabilizer buffer to each tube with mixing. Duplicate reactions were carried out for all samples and reagent blanks were

prepared by adding 0.1 ml sample to tubes containing substrate and stabilizer (i.e. in which reaction is terminated before the addition of enzyme). Within one hour the samples were read at 550 nm using a Hitachi-Perkin Elmer 139 spectrophotometer. As in the protein determinations, trial analyses were performed in order to find dilutions which would produce adequate colour within a reasonable incubation period.

Standard curves were prepared according to Manning et al. (1967). A stock phenolphthalein standard (1 mg/ml 50% ethanol) was used to prepare samples of 100 to 500 μg, and 0.1 ml aliquots of each were added to 5 (duplicate) substrate tubes (37.5°C). Following mixing, colour stabilizer was added (timing not required).

The resultant linear curve was used to obtain a slope conversion factor (s), equivalent to 60 μg phenolphthalein. The following calculation was then used to determine alkaline phosphatase specific activity: alkaline phosphatase sp.act. (μg phenolphthalein released/hr/mg duodenal protein) =

$$\frac{OD \times s \times df}{\text{Total Protein}} \times \frac{60}{t}$$

where OD = optical density @ 550 nm, df = dilution factor, and t = incubation time (min). (See Appendix II for sample calculation.) One unit of alkaline phosphatase activity liberates 1 μg of phenolphthalein in 60 min at

37°C from phenophthalein monophosphate at pH 10.1 under standard conditions (Manning *et al.*, 1967).

As a comparison, an additional enzyme curve was prepared from *E. coli* alkaline phosphatase (E.C. 3.1.3.1, Type III-R, Sigma). The standard, defined as containing 26 U/mg and 5.8 mg protein /ml, was used to prepare samples of 0.03 to 0.15 U/ml. From these, aliquots of 0.1 ml each were taken and analysed using the standard procedure (37 ± 0.5°C) for 10 minutes each. Comparison of the curves obtained provided additional indication that standard conditions were maintained during different runs. (See Appendix III for curve.)

The amount of variation introduced into the results via the biochemical methods used was determined and is reported in Appendix IV.

4. Statistical Analysis

The results of total protein, alkaline phosphatase specific activity, and weight determinations were analysed statistically using Duncan's New Multiple Range Test (Duncan, 1955, 1957; see Kirk, 1968). A Fortran program, developed for this test by D.L. Mallon (see Goldberg, 1971) and modified and entered on tape as DUNCAN (Fortran IV) by C.E. Shay was used. (See Appendix V).

IV Morphological Analysis

For morphological analysis, duodenal loops were excised (experiments performed *in vivo*) and immersed in ice cold half strength Karnovsky fixative, pH 7.4, (Karnovsky, 1965). After 5 to 10 minutes of fixation each loop was cut into smaller pieces and these were further fixed for 4 to 12 hours. Duodenal segments which were cultured *in vitro* were first pre-treated with 0.3 M sucrose (5 min @ 4°C) then fixed with 3% gluteraldehyde in 0.1 M sodium cacodylate buffer, containing $CaCl_2$ (0.1 M) and sucrose (0.3 M) pH 7.4 for 4 to 12 hours at 4°C. The fixation technique used for cultured material was developed by Buckley (1973) for embryonic cells in culture, and gave better structural preservation more consistently than did half strength Karnovsky fixative (as observed from sections examined with the light microscope). Fixation at 4°C produced better structural preservation than at room temperature.

Following fixation the tissues were rinsed in several changes of buffer (0.1 M cacodylate pH 7.4), then stored in the same buffer until further processing. Prior to post-fixation the samples were cut into small (1 - 2 mm^3) pieces and rinsed again in buffer. Segments of all samples were stored in cacodylate buffer (4°C) as reserve material. In the case of samples from the experiments performed *in vivo*, the material to be embedded

was taken from the anterior segment of the proximal loop (excluding the end centimeter). Material embedded from cultured segments came from their mid-portions.

The tissues were post-fixed 2 hr in 2% OsO_4 in 0.1 M cacodylate buffer or Millonig's buffer (Millonig, 1961) at 4°C. Following post-fixation the tissues were rinsed in buffer 10 min, then dehydrated through 10 min changes of: 30, 50, 70, and 90% ethyl alcohol at 4°C; and three changes of 100% ethyl alcohol, and two of propylene oxide at room temperature. The tissues were infiltrated through propylene oxide-resin mixture (1:1) to pure resin over a two day period (room temperature), then embedded in Araldite 502 resin (Ladd Research Industries) after Luft (1961) and Coulter (1967). Size 00 gelatin capsules and heat polymerization (Fisher Isotemp vacuum oven) were used.

Thick (1 - 2 μ) sections were cut using glass knives (LKB Ultrotome III), heat dried on glass slides, stained with 0.5% toluidine blue in 1% borax (sodium tetraborate, pH 11.0) by brief heating, rinsed with distilled water and dried. These sections were examined by light microscopy for orientation purposes prior to thin sectioning. Some of the sections were photographed using a Zeiss Photomicroscope on Kodak Panatomic X film (ASA 125).

Thin sections (approximately 60 - 80 nm) were cut using glass or diamond knives (Freidrich Dehmer, W. Germany) and picked up on uncoated 200 mesh copper grids. The sections were stained for 40 to 60 min with a saturated solution of uranyl acetate in 50% ethanol or methanol, then for 5 to 7 min using lead citrate according to Reynolds (1963). The grids were examined at 60 kv using a Siemans 1A electron microscope and photographed on Kodak sheet film (4489 or 4463).

Dimensions reported in the morphological descriptions are the means of 5 to 10 measurements on different cells and in different areas. These are intended only to indicate trends, as extensive quantification by means of measurements or counts was not performed.

RESULTS

I Biochemical Analysis

A Results of Experiments Performed *In Vivo*

1. General

In experiments performed *in vivo*, embryos were treated at approximately 16.5 days of development in the attempt to treat stage 42 embryos. At this time a few embryos were randomly sampled to confirm that stage 42 had in fact been reached. However, not all embryos reach the same stage at the same time (Hamburger and Hamilton, 1951), and at each sample time several stages were found both older and younger than the most numerous or 'correct' stage. (See Results A-5 concerning the distribution of developmental stages.)

As the stage of each embryo cannot be known for certain at the time of treatment, it was assumed that embryos are in each of the stages 41 through 44 for 24 hours, and in stage 45 for 48 hours or until hatching, as determined by Hamburger and Hamilton (1951). The stage distributions obtained appeared to support this assumption, and treatment did not appear to alter the general time course of development (Results A-5). Therefore, stage 43 embryos killed 24 hours after treatment

are assumed to have been in stage 42 at the time of treatment and so on.

The results of the *in vivo* experiments are dealt with in two ways: according to stage and according to time. In the staged results, the 'correct' or main stage is dealt with at each sample time and other stages are included only where they were encountered in sufficient numbers to be included in statistical analyses ($n = 5$ or more). In the results pooled according to time, at each time the results for all stages encountered were pooled with the exceptions of normal and windowed embryos which were grouped only according to stage as they did not receive treatments. Therefore 48 hours after treatment, staged results for L-T_4 treated embryos would comprise only embryos which were stage 44 at that sample time, while pooled results would be comprised of all L-T_4 treated embryos found at that time regardless of stage. In presenting the results of statistical analyses, the level of signifigance is 5% ($p < 0.05$) unless otherwise stated.

2. Duodenal Protein Content

a. Stage

Total duodenal protein content was found to increase from 2.1 ± 0.1 to 5.0 ± 0.3 mg, from stages 41 to 45 (Table 3) in normal embryos. The largest increase in protein content was found to occur between

Table 3 Duodenal Alkaline Phosphatase Sp. Act., Protein Content, Fresh and Dry Weight, and Body Weight of Normal Stage 41 to 45 Embryos

	Stage 41	42	43	44	45
Alkaline Phosphatase Sp Act (μg/hr/mg)	477.0 ± 50.1 (9)	855.9 ± 62.9 (38)	1047.6 ± 97.3 (26)	1707.3 ± 143.3 (43)	3240.7 ± 488.3 (20)
Total Protein (mg)	2.1 ± 0.1	2.4 ± 0.1 (40)	2.8 ± 0.2	4.4 ± 0.2	5.0 ± 0.3
Fresh Weight (mg)	32.1 ± 0.9 (8)	35.8 ± 0.7	41.0 ± 1.1	67.7 ± 2.1	81.7 ± 2.9
Dry Weight (mg)	4.3 ± 0.2	5.4 ± 0.1 (36)	6.1 ± 0.2	9.5 ± 0.3	13.9 ± 0.6
Body Weight (g)	13.8 ± 0.5	15.0 ± 0.3 (29)	16.7 ± 0.4 (17)	22.6 ± 0.4 (31)	27.1 ± 0.4

Results underlined by same line are not significantly different (Duncan's New Multiple Range test) at 5% level of significance.
± SEM, n in parentheses.

stages 43 and 44. In order to obtain an indication of the magnitude of change from stage to stage, the normal protein contents of each stage were compared using the Duncan's New Multiple Range comparison. This analysis revealed that stages 41 through 43 did not differ significantly from each other, while stages 44 and 45 differed significantly from each other as well as from the other three stages.

Twenty-four hours after treatment with L-T_4, stage 43 embryos had significantly greater total duodenal protein contents than did normal, windowed, and saline-treated embryos of the same stage (Table 4, Fig. 1). The protein contents of the L-T_4 and D-T_4 treated groups did not differ significantly, and the latter did not differ significantly from the other stage 43 groups (24 hr).

Stage 41 and 42 embryos were also found at 24 hours. The protein content of stage 42 D-T_4 treated embryos was significantly greater than that of stage 42 normal embryos, but did not differ from that of the stage 42 L-T_4 treated embryos (Table 4). The latter group and the remaining stage 42 groups did not differ significantly from each other. The few stage 44 embryos found at 24 hours were in insufficient numbers for comparison.

Tables 4.- 7. Results of experiments performed in vivo; staged and time pooled results. In all tables: values are shown ± SEM (standard error of the mean); group size (n) is indicated in parentheses accompanying the value in table body; values underlined by the same line are not significantly different using Duncan's New Multiple range analysis at the 5% level of significance ($p > 0.05$); values not underlined by the same line but in the same row of table are significantly different at this level. Comparisons are made only in the same row, except in the case of staged results where groups of the same stage occur at different times, e.g. stage 43 at 24 and 48 hrs. Here all values in first row (containing all treatment groups) are compared, then second row (incomplete) values entered into comparison with first row values. Results are entered as lines under second row, showing relationships of these values to values in upper row. N - normal, W - windowed, S - saline-treated, D - D-T_4 treated, L - L-T_4 treated.

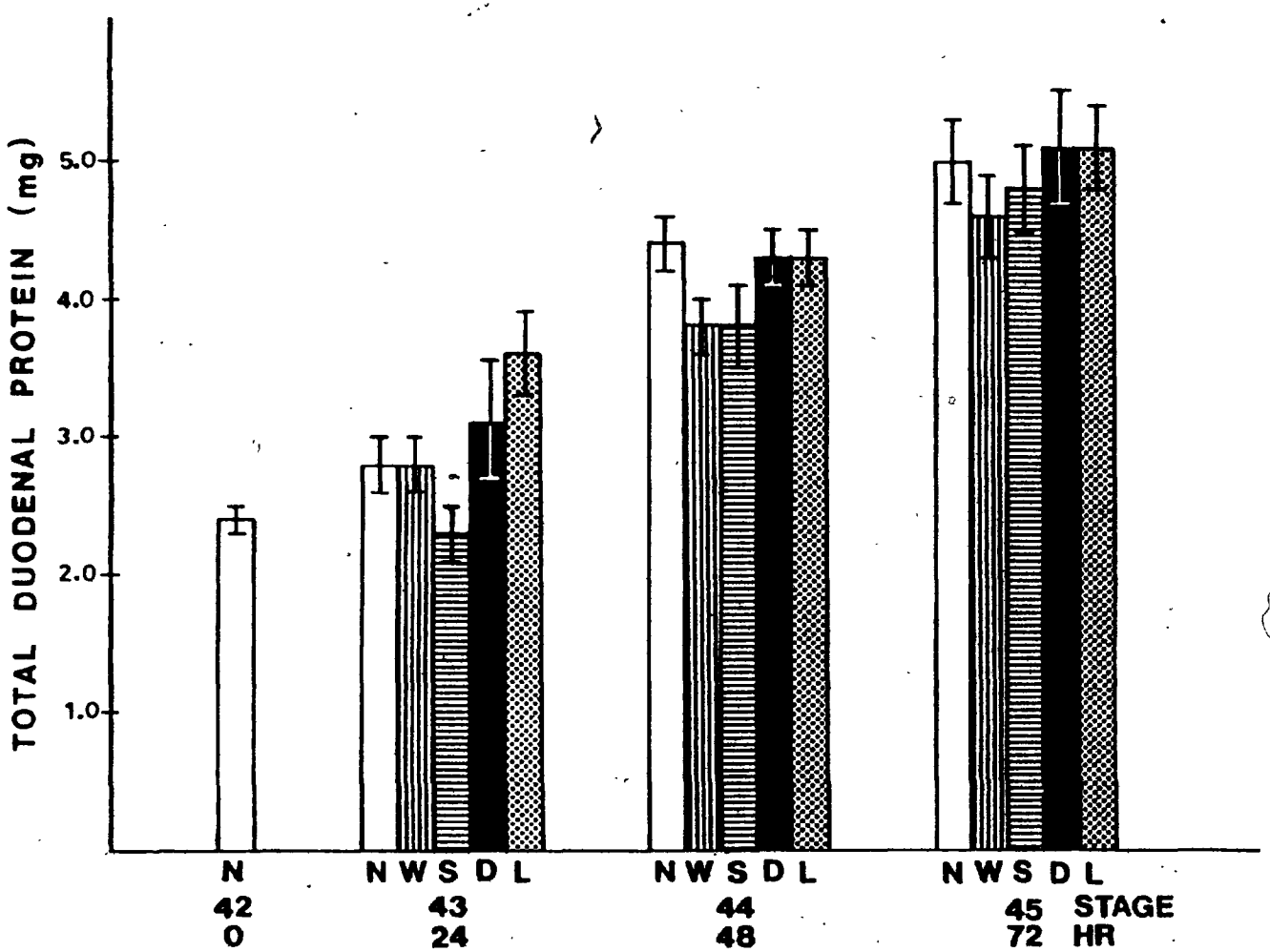
Table 4 Total Protein Content of Duodenal Homogenates - Staged Results

Time After Treatment - Stage	Group Total Protein Content (mg)						
24 Hr - 41,42	41S (4) 1.4 ± 0.2	41N (9) 2.1 ± 0.1	42S (8) 2.2 ± 0.2	42W (11) 2.3 ± 0.2	42N (40) 2.4 ± 0.1	42L (5) 2.5 ± 0.4	42D (8) 3.0 ± 0.2
24 Hr - 43	S (8) 2.3 ± 0.2	W (20) 2.8 ± 0.2	N (26) 2.8 ± 0.2	D (10) 3.1 ± 0.4	L (19) 3.6 ± 0.3		
48 Hr - 43					D (5) 3.8 ± 0.3	L (8) 3.8 ± 0.4	S (6) 4.0 ± 0.4
48 Hr - 44	S (16) 3.8 ± 0.3	W (14) 3.8 ± 0.2		L (20) 4.3 ± 0.2	D (19) 4.3 ± 0.2	N (43) 4.4 ± 0.2	
72 Hr - 44		L (10) 3.8 ± 0.2					
72 Hr - 45	W (9) 4.6 ± 0.3	S (14) 4.8 ± 0.3		N (20) 5.0 ± 0.3	D (15) 5.1 ± 0.4	L (16) 5.1 ± 0.3	
48 Hr - 45	L (18) 4.0 ± 0.2						

Figures

In all figures of results: bars represent SEM; n of each group is found in appropriate table referred to in legend; N - Normal, W - windowed, S - Saline, D - D-T_4, L - L-T_4.

Fig. 1. Total protein content (mg) of duodenal homogenates. (Refer to Table 4.) Treatment time (0 hr) is represented by stage 42 normal protein content. Only the results of most frequently encountered stage are shown at each time following treatment (24 to 72 hr).


TOTAL DUODENAL PROTEIN (mg)
5.0
4.0
3.0
2.0
1.0
N
42
0
N W S D L
43
24
N W S D L
44
48
N W S D L
45
72
STAGE
HR

Forty-eight hours after treatment, none of the stage 44 groups differed significantly with respect to duodenal protein content (Table 4, Fig. 1). Stage 43 embryos found at this time (L-T_4, D-T_4, and saline-treated) had protein levels significantly greater than did normal stage 43 embryos but not different from stage 43, 24 hr L-T_4 treated embryos (Table 4). The protein contents of stage 45 embryos treated 48 hours previously with L-T_4 did not differ significantly from stage 44 normal and other embryos, but had protein contents significantly lower than normal stage 45 embryos (but not different from stage 45 windowed and saline-treated embryos).

Seventy-two hours after treatment, all stage 45 groups had similar duodenal protein contents. Stage 44 embryos treated 72 hours previously with L-T_4 had protein contents significantly less than the 72 hour stage 45 groups, but not significantly different from the other stage 44 groups found at 48 hours.

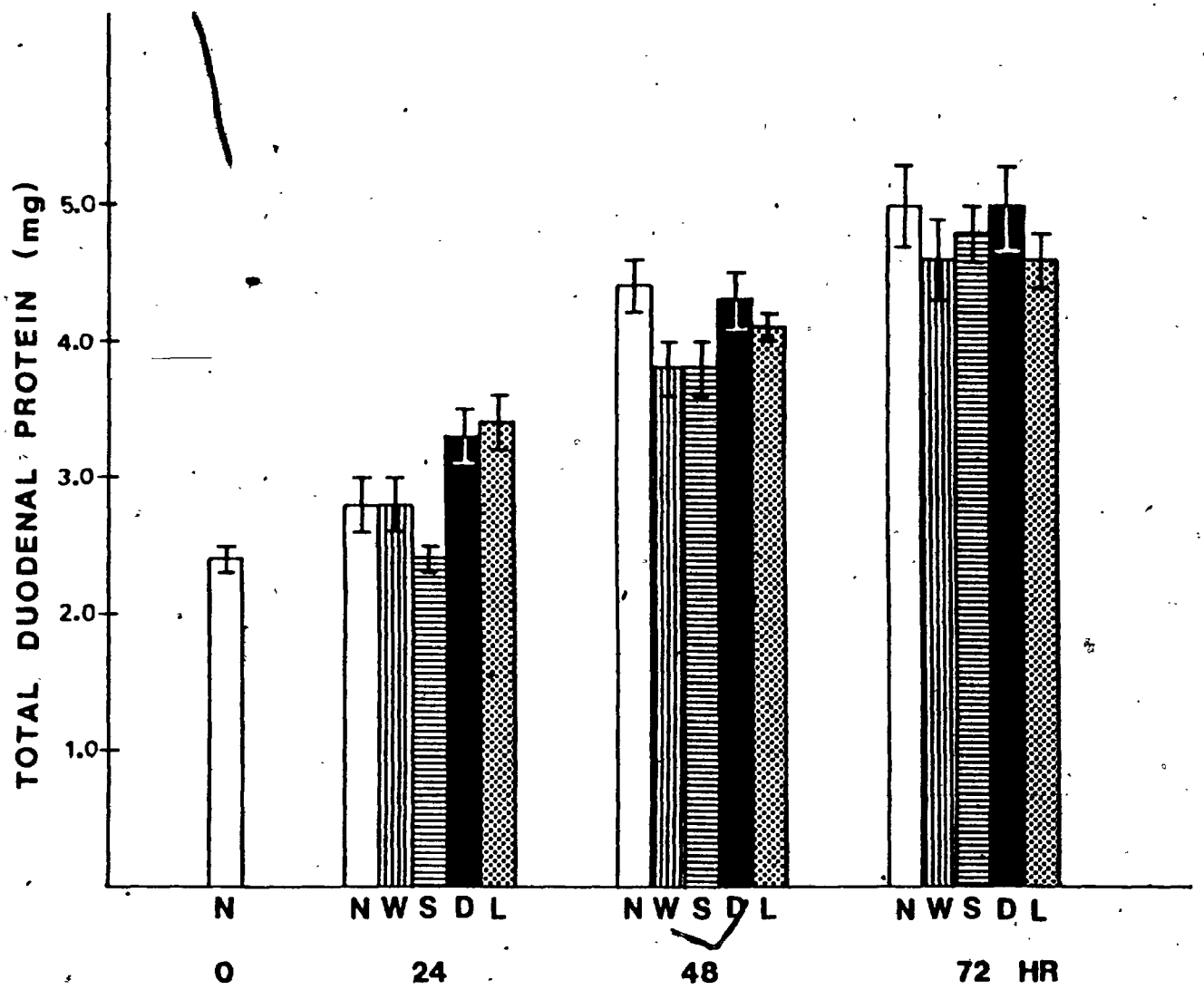
b. Time Pool

When the results of the duodenal protein determinations were pooled according to time (i.e. all stages at one sample time pooled), at 24 hours the L-T_4 treated group's protein content was significatnly greater than the windowed and saline groups' but not greater than the D-T_4 and normal groups' (Table 5, Fig. 2). At 48 and

Table 5 Total Protein Content of Duodenal Homogenates - Pooled According to Time

Time After Treatment (hr)	Treatment Total Protein Content (mg)				
24	S (16) 2.4 ± 0.1	W (20) 2.8 ± 0.2	N (26) 2.8 ± 0.2	D (20) 3.3 ± 0.2	L (24) 3.4 ± 0.2
48	W (14) 3.8 ± 0.2	S (24) 3.8 ± 0.2	L (46) 4.1 ± 0.1	D (26) 4.3 ± 0.2	N (43) 4.4 ± 0.2
72	W (9) 4.6 ± 0.3	L (27) 4.6 ± 0.2	S (16) 4.8 ± 0.2	D (17) 5.0 ± 0.3	N (20) 5.0 ± 0.3

Fig. 2. Total Protein content (mg) of duodenal homogenates, results pooled according to time. (Refer to Table 5.) Treatment time (0 hr) is represented by stage 42 normal. Normal and windowed groups are comprised only of one stage at each time, other groups are pooled from all stages at one time.


TOTAL DUODENAL PROTEIN (mg)
5.0
4.0
3.0
2.0
1.0
N
N W S D L
N W S D L
N W S D L
0
24
48
72 HR

72 hours following treatment, no significant differences in protein content were found among any of the time-pooled groups (Table 5, Fig. 2).

3. Alkaline Phosphatase Specific Activity

a. Stage

Duodenal alkaline phosphatase specific activity (μg/hr/mg duodenal protein) increased from 477.0 ± 50.1 at stage 41 to 3240.7 ± 488.3 at stage 45 (Table 3). Stages 41 through 43 did not differ significantly from each other, while stages 44 and 45 differed significantly from each other and from the younger groups. The difference between stages 44 and 45 remained significant at the 1% level ($p<0.01$), indicating the magnitude of the increase at this time, greater than the increases between each pair of younger stages.

Twenty-four hours after treatment, no significant differences in duodenal alkaline phosphatase specific activity were seen among any of the stage 43 groups (Table 6, Fig. 3). Neither were the stage 42 embryos which had been treated 24 hours previously significantly different from normal stage 42 embryos.

Forty-eight hours after injection, the alkaline phosphatase activity of the stage 44 L-T_4 treated duodena had increased greatly, and was significantly greater than the other stage 44 (48 hr) activities

Table 6

Alkaline Phosphatase Sp. Act. of Duodenal Homogenates
Staged Results

Time After Treatment - Stage	Treatment - Alkaline Phosphatase Sp. Act. (μg/hr/mg)						
	41N (9)	41S (4)	42N (38)	42S (8)	42D (8)	42W (11)	42L (5)
24 Hr - 41,42	477.0 ± 50.1	840.9 ±212.8	856.0 ± 62.9	1026.7 ± 316.5	1152.9 ± 264.5	1290.6 ± 114.7	1356.9 ± 374.3
	N (26)	S (8)	W (20)	D (10)	L (16)		
24 Hr - 43	1047.6 ± 97.3	1185.8 ± 24[illegible].9	1500.2 ± 172.2	1508.6 ± 351.0	1584.6 ± 233.1		
		S (7)		D (5)	L (8)		
48 Hr - 43	—	1448.3 ± 265.5		1604.8 ± 514.7	3362.9 ±1313.4		
	S (16)	D (19)	W (14)	N (43)	L (20)		
48 Hr - 44	1277.5 ± 143.0	1454.7 ± 275.8	1526.1 ± 251.6	1707.3 ± 143.3	3444.8 ± 343.2		
			L (10)				
72 Hr - 44			1697.6 ± 207.1				
	S (17)	N (20)	D (15)	W (9)	L (16)		
72 Hr - 45	2626.9 ± 380.1	3240.8 ± 488.3	3737.8 ± 918.9	3839.8 ±1330.8	5059.7 ± 794.4		
							L (18)
48 Hr - 45							6012.0 ±1103.2

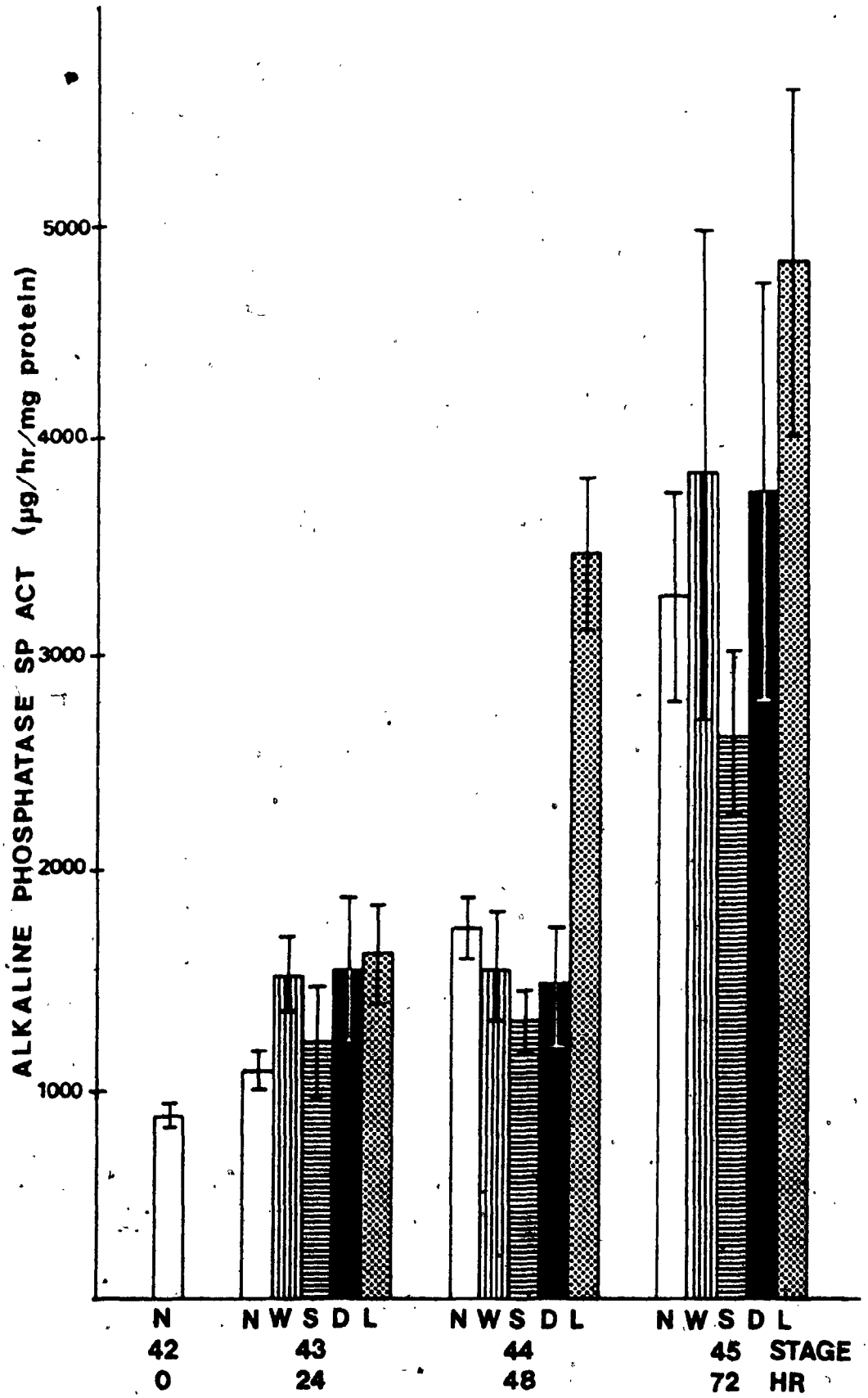
($p < 0.01$; Table 6, Fig. 3). Stage 43 and 45 embryos which had been treated 48 hours previously with L-T_4 also showed elevated phosphatase activities. The alkaline phosphatase activity of the stage 43 group was nearly as great as that of the 48 hr stage 44 group (Table 6), and was significantly greater than the other stage 43 (24 and 48 hr) groups.

Stage 45 L-T_4 treated embryos still possessed the largest duodenal alkaline phosphatase specific activities, compared to embryos of the same stage, 72 hours after treatment (Table 6, Fig. 3). However, none of the stage 45 72 hour groups differed significantly from any other with respect to phosphatase activity, even when the windowed group, which showed the largest variation, was omitted from the comparison. By this time the phosphatase activities of the control stage 45 groups had reached the levels the stage 44, L-T_4 treated embryos had attained 24 hours previously. Stage 45 embryos which had been treated 48 hours previously with L-T_4 had higher phosphatase levels than embryos of the same stage and treatment after 72 hours (Table 6). While these two groups did not differ significantly from each other, the former did differ significantly from the remaining stage 45, 72 hour groups. Stage 44 embryos which had received L-T_4 treatment 72 hours previously had phosphatase activities comparable to those of the stage 44 controls at 48 hours.

Table 7 Alkaline Phosphatase Sp. Act. of Duodenal Homogenates - Pooled According to Time

Time After Treatment (hr)	Treatment				
	Alkaline Phosphatase Sp. Act. (μg/hr/mg)				
24	N (26) 1047.6 ± 97.3	S (16) 1106.2 ± 195.0	D (19) 1453.2 ± 236.8	W (20) 1500.2 ± 172.2	L (24) 1809.1 ± 253.7
48	S (24) 1353.3 ± 122.8	D (26) 1498.5 ± 220.5	W (14) 1526.1 ± 251.6	N (43) 1707.3 ± 143.3	L (46) 4435.1 ± 532.7
72	S (16) 2491.2 ± 374.1	N (20) 3240.8 ± 488.3	D (17) 3490.0 ± 830.5	L (27) 3668.0 ± 574.0	W (9) 3839.8 ±1330.9

Fig. 3. Alkaline phosphatase specific activity of duodenal homogenates (μg/hr/mg). (Refer to Table 6.) Treatment time (0 hr) is represented by stage 42 normal. Only the results of most frequently encountered stage are shown at each time following treatment.


ALKALINE PHOSPHATASE SP ACT (µg/hr/mg protein)
5000
4000
3000
2000
1000
N
42
0
N W S D L
43
24
N W S D L
44
48
N W S D L
45
72
STAGE
HR

b. Time Pool

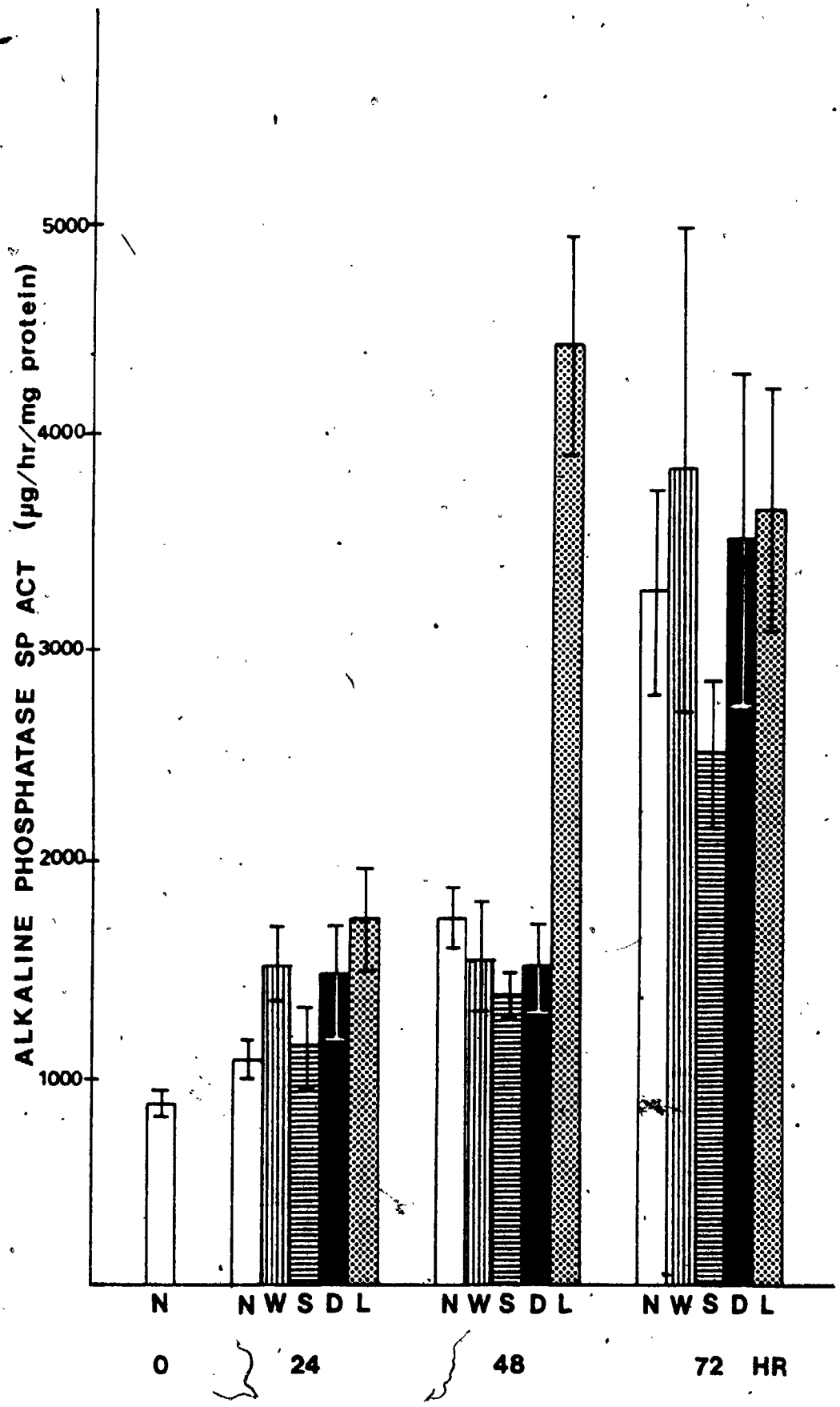
When sample results were pooled according to time without regard to stage, at 24 hours the alkaline phosphatase specific activity of the L-T_4 treated embryos remained the largest of the pooled groups (Table 7, Fig. 4). At this time the activity of the L-T_4 treated group was significantly larger than those of the saline-treated and normal groups, but not significantly different from the windowed and D-T_4 treated groups.

Forty-eight hours after treatment, the activity of the L-T_4 pooled group remained significantly greater than all other groups at the same time ($p<0.01$). At seventy-two hours no significant differences were found among any of the time-pooled group's phosphatase activities, and the L-T_4 treated value was no longer the largest or very different from the controls (Table 7, (Fig. 4).

4. Weights

Duodenal fresh and dry weights and embryonic body weight all increased from stages 41 to 45 (Table 3). Detailed analyses of all weights following treatment were performed, and these results are found in Appendices VI and VII. With L-T_4 treatment there were tendencies

Fig. 4. Alkaline phpsphatase specific activity (μg/hr/mg) of duodenal homogenates, results pooled according to time. (Refer to Table 7.) Treatment time (0 hr) is represented by stage 42 normal specific activity. Normal and windowed groups are comprised only of one stage at each time; other groups are pooled from all stages at a particular time.


ALKALINE PHOSPHATASE SP ACT (µg/hr/mg protein)
5000
4000
3000
2000
1000
N
N W S D L
N W S D L
N W S D L
0
24
48
72 HR

(i.e. significant differences compared to one or more control groups) towards increased duodenal fresh weight at 24 hr, and increased duodenal dry weights at all times. Body weight tended to be greater 24 hr following L-T_4 treatment. All windowed groups had body weights significantly less than those of the intact group at 72 hr.

5. Stages of Development

The distribution of developmental stages in each treatment group was noted at each sample time. At the time of treatment (day 16.5), of 256 embryos sampled, 9.8% were stage 41, 60.5% stage 42, and 29.7% stage 43 (Table 8, Fig. 5). Twenty-four, 48 and 72 hours following treatment, the stages which comprised the largest portion of any experimental group were 43, 44, and 45 respectively (Table 8, Fig. 5).

Stage 45 normally lasts until hatching or about 48 hours following stage 44 (Hamburger and Hamilton, 1951). During this time the embryos begin to pip (chip open) their shells, and while this activity does not indicate the exact time of hatching it is a component of pre-hatching activity. Therefore embryos which had begun to pip their shells were categorized as a group apart from the other stage 45 embryos for comparison of stage distribution. In comparison with the other groups, more

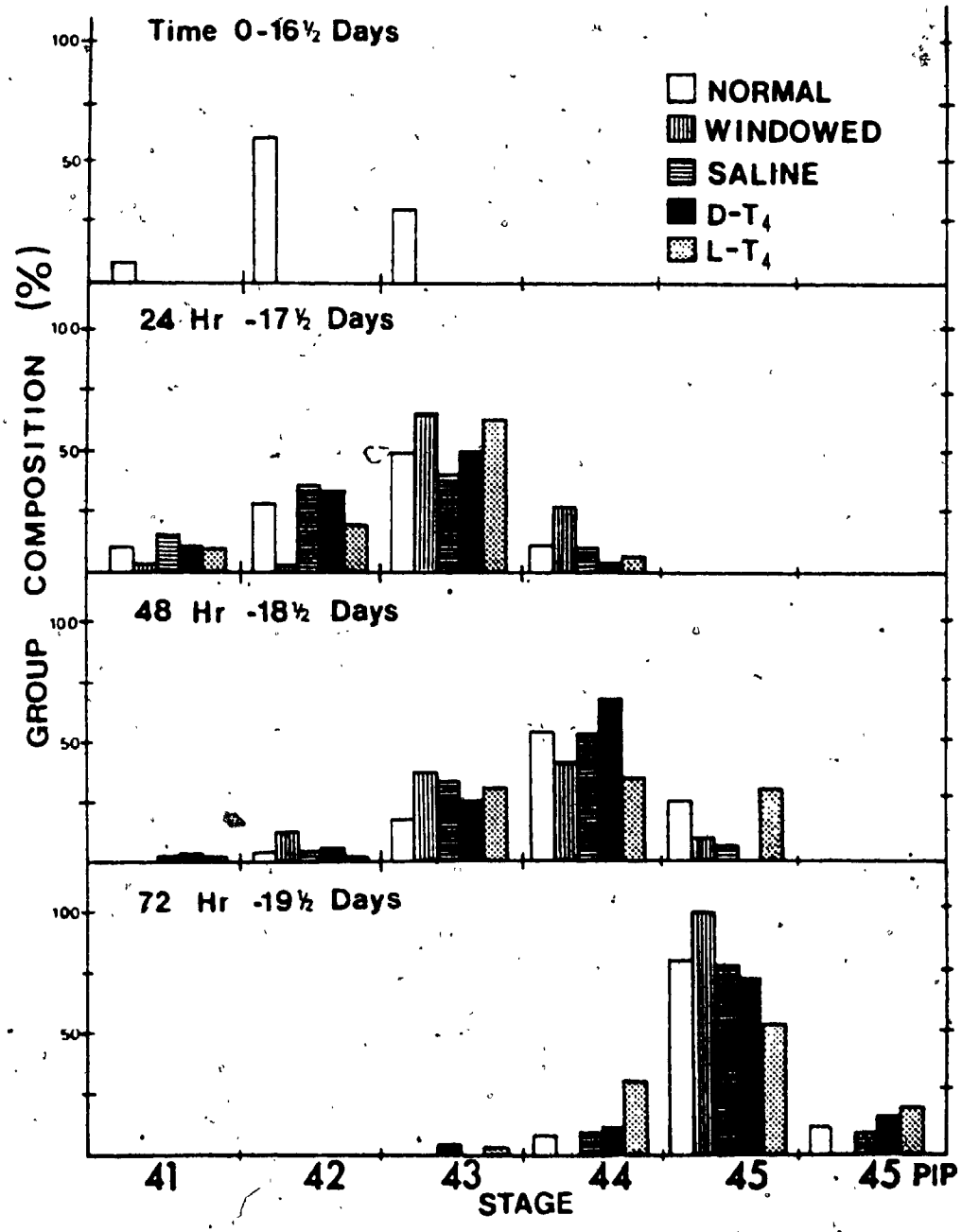
Table 8

Distribution of Developmental Stages

Time	Stage	Treatment Group N	W	S	D	L
0	41	25 (9.8)	-	-	-	-
	42	155 (60.5)	13 (76.5)	-	-	-
	43	76 (29.7)	4 (23.5)	-	-	-
24	41	9 (11.3)	1 (3.4)	6 (15.0)	4 (10.5)	4 (9.1)
	42	20 (25.3)	1 (3.4)	15 (37.5)	13 (34.1)	9 (20.4)
	43	39 (49.4)	19 (65.5)	16 (40.0)	19 (50.0)	28 (63.6)
	44	11 (13.9)	8 (27.6)	3 (7.5)	2 (5.3)	3 (6.8)
48	41	-	-	1 (2.1)	1 (2.3)	1 (1.5)
	42	2 (2.8)	6 (11.8)	2 (4.2)	2 (4.6)	1 (1.5)
	43	13 (18.3)	19 (37.2)	16 (34.0)	11 (25.6)	21 (31.3)
	44	38 (53.5)	21 (41.2)	25 (53.2)	29 (67.4)	24 (35.8)
	45	18 (25.3)	5 (9.8)	3 (6.4)	-	20 (29.8)
72	43	-	-	1 (4.3)	-	1 (2.8)
	44	2 (7.7)	-	2 (8.7)	2 (10.5)	10 (28.6)
	45	21 (80.8)	9 (100.0)	18 (78.3)	14 (73.7)	18 (51.4)
	45PIP	3 (11.5)	-	2 (8.7)	3 (15.8)	6 (17.1)

No embryos were found in stages indicated by -.
Percentage of embryos occurring in each stage at one time (for a single treatment) are shown in parentheses.

Fig. 5. Distribution of developmental stages at time of treatment (time 0) and subsequent 24 hour intervals to 72 hours following treatment. (Refer to Table 8.) At each time the number of individuals in each stage is expressed as a percentage of the total members of that treatment group occurring at that time only. Stages are according to Hamburger and Hamilton (1951), with the modification of dividing stage 45 to group stage 45 embryos which had pipped their shells (45 PIP) separately from stage 45 embryos which had not pipped (45).


Time 0-16½ Days
NORMAL
WINDOWED
SALINE
D-T4
L-T4
24 Hr -17½ Days
48 Hr -18½ Days
72 Hr -19½ Days
GROUP COMPOSITION (%)
100
50
41
42
43
44
45
45 PIP
STAGE

embryos treated with L-T_4 had pipped their shells at 72 hours (Table 8, Fig. 5), however the windowed group was the smallest in size.

The stage distributions at each time and treatment were compared and found to be within acceptable (95%) limits. Therefore significant changes in stage distribution were not produced within the duration of the experiment by any treatment.

6. Yolk Sac Retraction

In Hamburger and Hamilton's stages of normal development (1951), retraction of the yolk sac normally begins during stage 45, and by the time of hatching (stage 46) withdrawl is complete. Yolk sac retraction, i.e. the presence of condensation of the yolk and obvious withdrawl of the sac and its contents into the body cavity, was noted at the sampling times. No attempt was made to quantify the relative degree of retraction, and any obvious amount was recorded as indicating that this process had begun.

Yolk sac retraction was observed to begin earliest in the embryos treated with L-T_4. At 48 hours, 50% of the stage 44 L-T_4 treated embryos displayed some degree of retraction, in contrast to the absence of evidence of this process in all other stage 44 embryos

Table 9 Yolk Sac Retraction

Time After Treatment	Stage	Treatment	# Embryos Started Retraction/# in Group	% Started Retraction
48	43	L	1/8	12.5
	44	L	10/20	50.0
	45	L	8/18	44.4
72	44	L	4/10	40.0
	45	N	11/20	55.0
	45	W	0/9	0.0
	45	S	9/15	60.0
	45	D	15/15	100.0
	45	L	16/16	100.0

No retraction was found at times and stages which are not shown in table.

(Table 9). Stage 45 embryos treated 48 hours previously with L-T_4 had begun retraction in 8 out of 18 embryos.

At 72 hours, all stage 45 L-T_4 and D-T_4 treated embryos, and more than half of all normal and saline-treated embryos, had begun to retract their yolk sacs (Table 9). None of the windowed stage 45 embryos had started to retract their yolk sacs at this time.

7. Mortality

Mortality in each group was determined from the time of treatment. All windowed eggs were inspected visually as to the condition of the blood vessels beneath the window, and only live embryos were used to give rise to the four windowed groups. Normal (unwindowed) eggs were candled, and while not entirely accurate, this procedure served to remove most of the dead embryos from the group. After treatment, all embryos were left undisturbed until the appropriate sampling time. Thus mortality at each time was derived using only the groups then sampled, i.e. embryos were not examined before the time at which they were to be killed, nor were mortalities accumulated from one time to the next. Mortality was determined only in relation to time, since the stages of embryos which were already dead were not determined due the rapidity of decomposition.

Table 10 Mortality

Time After Treatment (hr)	Treatment Number Dead/ Number in Group				
	N	W	S	D	L
24	10/62 (16.1)	8/33 (24.2)	6/35 (17.1)	12/48 (25.0)	13/54 (24.0)
48	19/37 (51.3)	3/36 (11.1)	4/46 (8.7)	6/58 (10.3)	85/172 (49.4)
72	22/45 (48.9)	10/19 (52.6)	14/43 (32.6)	33/64 (51.6)	100/134 (74.6)

Percentage of dead embryos in each group is indicated in parentheses.

All embryos were alive at time of treatment (0 hr) as determined by observation through window, or by candling in case of normal (unwindowed) embryos.

Twenty-four hours after treatment, 16 to 17% of the normal and saline-treated embryos were found dead, compared to about 25% of each of the other groups (Table 10). Forty-eight hours after treatment, mortality ranged from 8.7% in the saline-treated group to 51.3% in the normal group. Twenty-four hours later, at 72 hours, the lowest mortality was found in the saline-treated group (32.6%) and the highest in the L-T_4 treated group (74.6%).

The proportion of malformed embryos was constant in all groups, approximately 1.5%. These embryos were alive at the time of sampling, but exhibited externally visible malformations, e.g. protruding organs, malformed limbs, gross underdevelopment, therefore were discarded.

B Results of Experiments Performed *In Vitro*

1. General

In the experiments performed *in vitro*, duodenal quarters were explanted from stage 42 embryos and cultured for 48 hours in the presence of media (McCoy's 5A) containing L-T_4, D-T_4, or saline vehicle, with or without added bovine serum albumin (BSA). Duodenal alkaline phosphatase specific activity had been found to vary greatly among individual duodena (Results A-3),

as reflected in large Standard Errors of the Mean. Therefore, quarters from the same duodenum were cultured with different media to offset the influence of individual variation on the culture results. Preliminary determinations revealed that the duodenal quarters were sufficiently similar in phosphatase and other characteristics to allow such comparisons to be made among them. (Refer to Appendix VIII.) No quarter from any position in the duodenal loop was found to respond differently from the other quarters in culture, either to specific additions to the media, or to the general conditions of normal culture. Therefore the results do not make reference to the portion of the duodenum from which each quarter was derived. The results obtained from cultures with D-T_4 were found not to differ with the hormone concentrations used: 14 ng/ml or 14 ng/100 ml. Therefore these results were pooled as a single D-T_4 group. The results of preliminary experiments involving the explant of stages other than 42, cultured for periods other than 48 hr, or using other hormone concentrations are presented in Appendices IX to XI; stage 42 weight data following 48 hr in culture is included in Appendix X.

2. Total Protein Content of Duodenal Quarters

Following 48 hr of culture *in vitro*, duodenal quarters cultured with D-T_4 exhibited the largest protein

contents in both sets of cultures (with and without BSA; Table 11, Fig. 6). In both cases these values were significantly greater than those determined for explants cultured with normal or L-T_4 containing media. In cultures without BSA, explants cultured with 14 ng L-T_4/100 ml of medium had significantly smaller protein contents than those cultured in normal medium (Table 11, Fig. 6). The protein contents of explants cultured with 14 ng L-T_4/ml of medium did not differ significantly from those of either of the two aforementioned groups. In cultures containing BSA, no significant differences in protein content appeared among the groups not containing D-T_4.

When the results of cultures with and without BSA were all combined in the same Duncan's New Multiple Range analysis (Table 11), the protein content of cultures with D-T_4 and BSA no longer were significantly different from those cultured in normal media containing BSA. None of the other significant differences changed. The two sets of cultures containing D-T_4 did not differ significantly in protein content of the quarters.

Compared to the protein contents of normal stage 42 quarters (Table A-11), the values determined for stage 42 explants cultured 48 hours are low. Similarly, multiplication of the highest protein content encountered in these cultures by four to give an estimate

Table 11 Protein Content and Alkaline Phosphatase Specific Activity of Homogenates of Stage 42 Duodenal Quarters Cultured 48 Hours In Vitro

	No BSA				With BSA			
lkaline hosphatase p Act (µg/hr/mg)	D (9) 803.0 ± 103.2	N (24) 1023.3 ± 72.1	L(14/1) (16) 1029.3 ± 149.0	L(14/100) (27) 1598.7 ± 187.7	D (22) 678.1 ± 43.4	L(14/100) (21) 1088.9 ± 114.6	N (23) 1201.1 ± 114.6	L(14/1) (22) 1411.9 ± 116.7
otal rotein (mg)	L(14/100) 0.23 ± 0.02	N 0.23 ± 0.02	L(14/1) 0.26 ± 0.03	D 0.38 ± 0.02	L(14/100) 0.23 ± 0.02	L(14/1) 0.28 ± 0.02	N 0.30 ± 0.02	D 0.38 ± 0.02

he lines underscoring the above data indicate the results of Duncan's comparisons treating ultures with and without BSA separately. The results when all groups are combined in the same omparison are shown below.

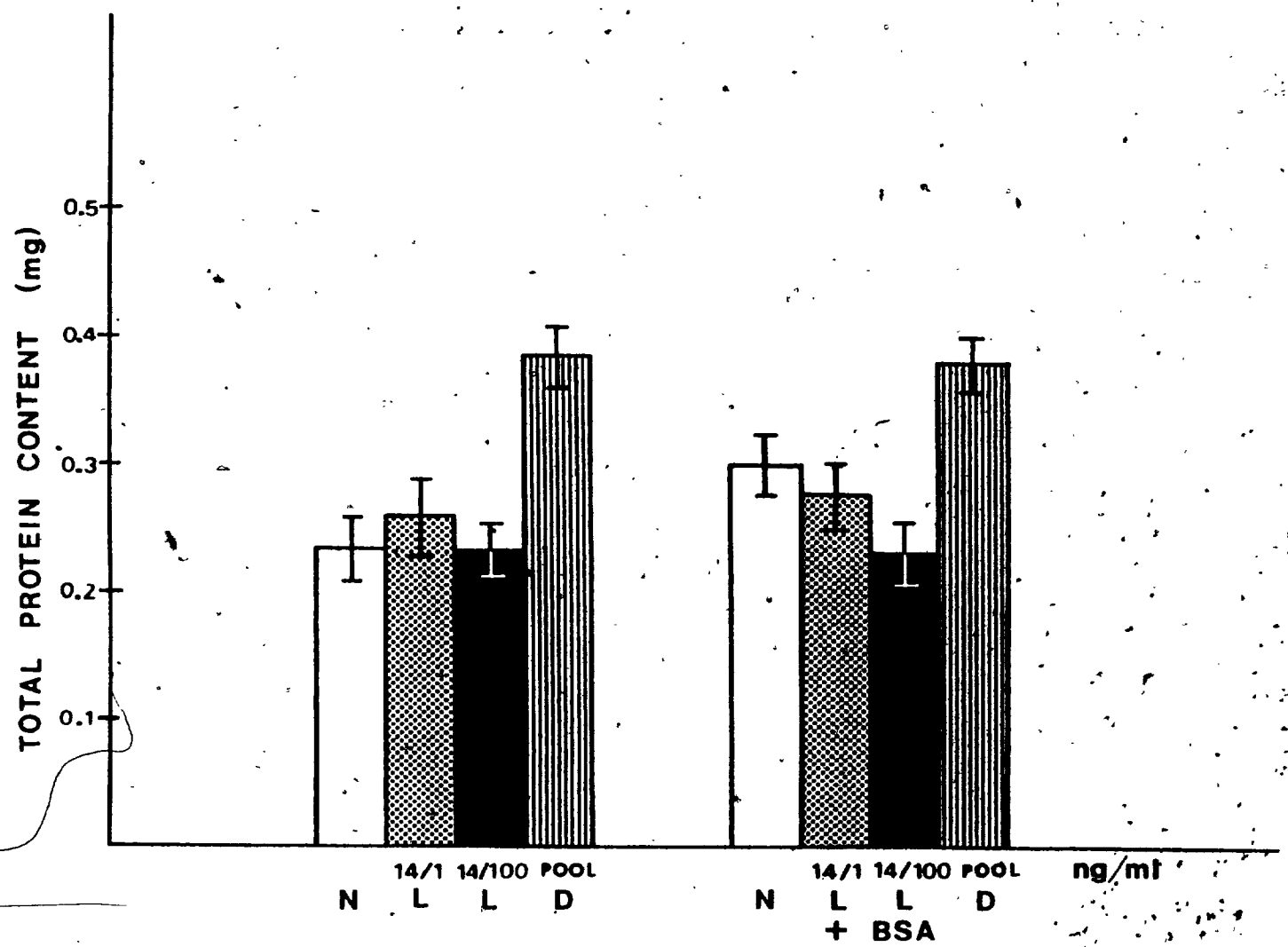
lkaline Phosphatase Specific Activity (µg/hr/mg)

+BSA D N L(14/1) L(14/100)+BSA N+BSA L(14/1)+BSA L(14/100)

rotein Content (mg)

(14/100)+BSA L(14/100) N L(14/1) L(14/100) N+BSA D+BSA D

Fig. 6. Total protein content (mg) of stage 42 duodenal quarters, cultured *in vitro* for 48 hr. L-T_4 concentrations were 14 ng/ml and 14 ng/100 ml. Results using similar D-T_4 concentrations were pooled as a single group. (Refer to Table 11.)


TOTAL PROTEIN CONTENT (mg)
0.5
0.4
0.3
0.2
0.1
N
14/1 L
14/100 L
POOL D
N
14/1 L + BSA
14/100 L
POOL D
ng/ml

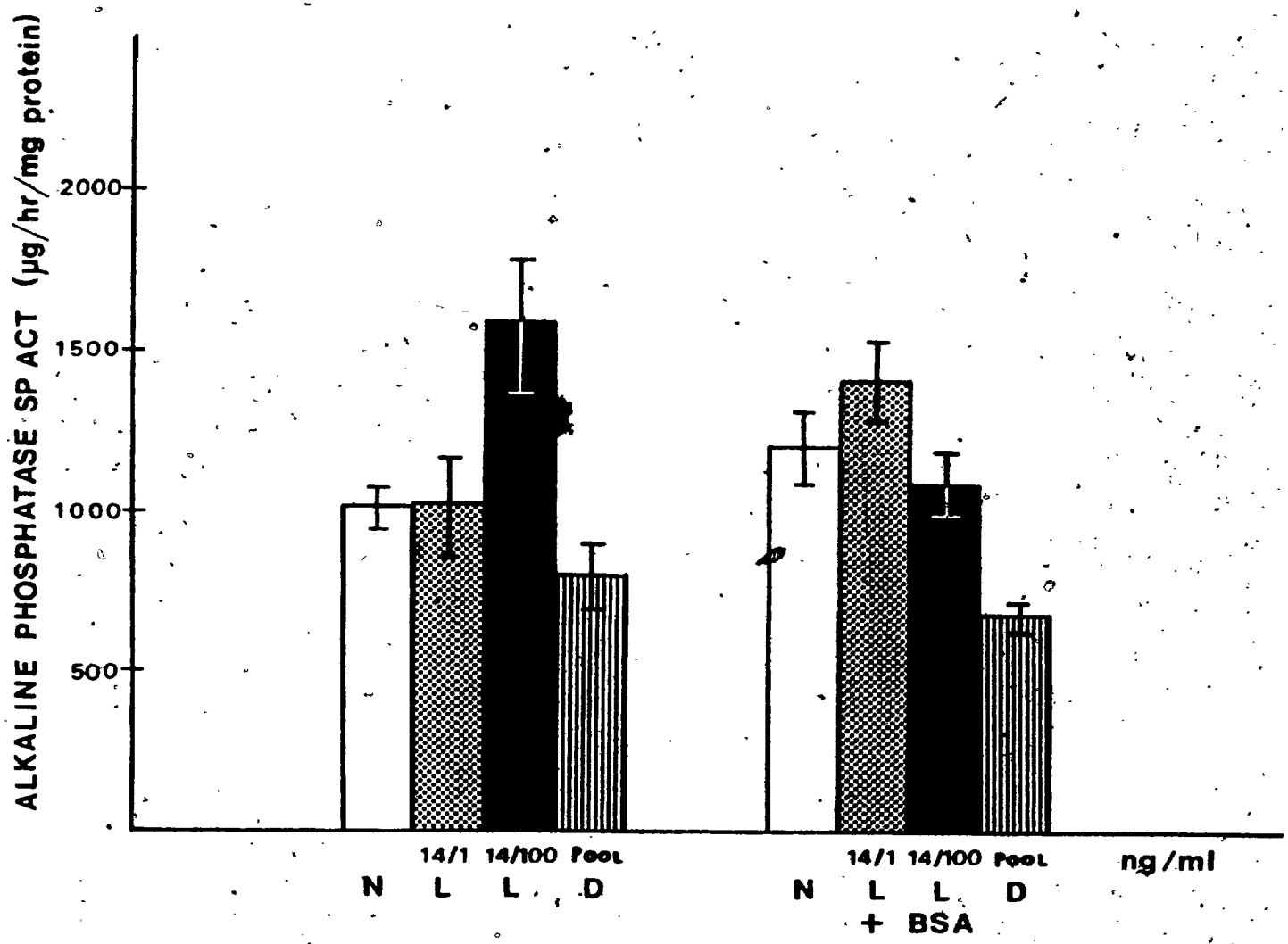
of total duodenal protein, gives a value of 1.54 mg, similar to the protein content of stage 41 saline-treated duodena (Table 4). Therefore, protein content of stage 42 explanted quarters appears to have decreased following 48 hours in culture.

3. Alkaline Phosphatase Specific Activity

After 48 hr of culture without BSA, the mean alkaline phosphatase specific activity of stage 42 duodenal quarters cultured with 14 ng L-T_4/100 ml was significantly greater than those of all other groups (Table 11, Fig. 7). The specific activities of the three other groups did not differ significantly from each other, and the lowest activity was determined for the group of explants cultured with D-T_4.

In cultures containing BSA, the greatest specific activity was shown by explants cultured with 14 ng L-T_4/ml of medium (Table 11, Fig. 7). However, the specific activity of the aforementioned group did not differ significantly from that of explants cultured with normal medium containing BSA, yet was significantly greater than the activity of explants cultured with 14 ng L-T_4/100 ml of medium. Again the lowest specific activities were shown by cultures containing D-T_4, however here the difference was significant compared to all other BSA containing groups.

Fig. 7. Alkaline phosphatase specific activity (μg/hr/mg protein) of stage 42 duodenal quarters cultured *in vitro* for 48 hr. L-T_4 concentrations were 14 ng/ml and 14 ng/100 ml. Results using similar D-T_4 concentrations were pooled as a single group. (Refer to Table 11.)


ALKALINE PHOSPHATASE SP ACT (µg/hr/mg protein)
2000
1500
1000
500
14/1 14/100 POOL
N L L D
14/1 14/100 POOL
N L L D
+ BSA
ng/ml

When all of the groups, with and without BSA, were included in the same statistical comparison, none of the previously described differences changed in significance (Table 11). The largest phosphatase activities did not differ significantly (14 ng L-T_4/100 ml and 14 ng L-T_4/ml + BSA). The alkaline phosphatase specific activity of explants cultured with D-T_4 and BSA did not differ significantly from those of the three groups without BSA which had the lowest activities (D-T_4, normal, and 14 ng L-T_4/ml) (Table 11).

The alkaline phosphatase specific activities determined after stage 42 explants had been cultured *in vitro* for 48 hr approximated those of stages 41 (D-T_4 containing cultures) to 43 (Table 6). The activities determined for stages 42 and 43 showed considerable overlap in the experiments performed *in vivo*, however the largest activities encountered in these cultures appear to be definitely within the stage 43 range. Therefore, alkaline phosphatase specific activity did not increase as greatly in duodena maintained in culture as in normal intact duodena.

II Morphological Analysis

A Introduction

During the course of experiments performed *in vivo* and *in vitro*, portions of duodena were fixed and embedded for subsequent microscopic observation (see Materials and Methods IV). In the former experiments the material examined was taken from the anterior segment of the proximal duodenal loop, and in the latter experiments from the mid-portions of the explants. Thick sections were observed using light microscopy for orientation prior to thin sectioning for electron microscopy.

The observations presented in the following sections deal mainly with the absorptive epithelial cells (although observations on goblet cells are presented) and pertain only to cells on the upper thirds of the villi, i.e. the most mature cells. Quantitative terms (e.g. "appeared more frequently") are often used descriptively for comparative purposes, but are only intended to indicate trends.

B Experiments Performed *In Vivo*

Preliminary observations showed no differences in duodenal morphology of normal, saline-treated, or windowed embryos, therefore the 'normal' morphology to be described pertains to all of these groups.

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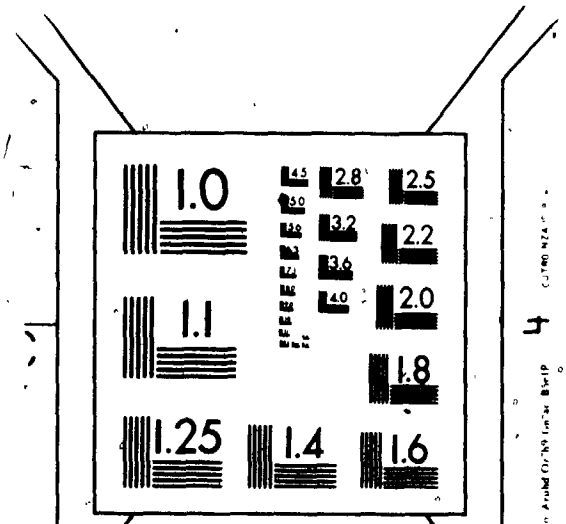


1. Light Microscopy

The villi of the stage 42 duodenum were covered by a simple cubiodal to low columnar epithelium (Fig. 8). With increasing stage the epithelial cells became progressively taller or more columnar and their apical cytoplasm (above the nucleus) enlarged (Figs. 8, 10, 14, 18). Epithelial cell height increased from approximately 12 μ at stage 42, to a maximum of 24 μ at stage 45, while cell width decreased from approximately 5 to 3 μ. At any stage examined, taller cells of more mature appearance than those lower on the villi were found near the villar tips. Mitoses were not observed in the upper regions of the villi.

At stage 42 a large nucleus possessing one or more distinct nucleoli occupied most of the internal area of the cuboidal absorptive cell (Fig. 9). With increasing stage the nucleus assumed a basal position and occupied a relatively smaller area of the cell as the apical cytoplasm increased in size (Figs. 10 to 22). Many cells contained large pale-staining, rounded inclusions [lipid droplets (Dobbins et al., 1967;Holman, 1969)], which filled the apical and/or basal cytoplasm, sometimes compressing the nuclei (Figs. 8 to 22). These inclusions were seen less frequently with increasing stage.

By stage 44 the brush border was visible as a distinct band on the cells' luminal surface (Figs. 14 to 22)

and many stage 44 and 45 cells exhibited pale-staining regions above their nuclei (Figs. 15, 22).

Goblet cells were seen more often at stage 45 than in the earlier stages. In sections stained with toluidine blue, the mucous of the goblet cells stained metachromatic pink. This facilitated the location of cells which had not yet formed a large mucous cup, however in the black and white photographs only goblet cells possessing large amounts of mucous can be readily discerned (Figs. 19, 21).

Using light microscopy, the duodenal epithelium appeared similar in normal and T_4 treated embryos of the same stage in any of the stages examined.

2. Electron Microscopy

a. Absorptive Cells

Form

When observed electron microscopically, the overall form of the absorptive epithelial cells and the changes in appearance from cuboidal to columnar agreed with the light microscopic observations. At stage 42, interdigitations between apposing cells were most extensive at the apical and nuclear levels (Fig. 23), and often long sections of lateral cell membrane were seen which did not interdigitate with adjacent cells, but did

form tight junctions with them. At later stages interdigitation of the lateral membrane became quite extensive (Fig. 32). At all stages junctional complexes were present between adjacent cells in a juxtaluminal position (Fig. 23), the apical surface formed microvilli, and the basal surface rested on a thin filamentous basement lamina (Fig. 23a). Intercellular spaces were more extensive at stages 44 and 45 than 42 and 43, and contained slightly electron dense material (Fig. 34).

Brush Border

The development of the brush border has previously been described in detail (Overton and Shoup, 1964; Ono, 1973) and the observations made here confirm the previous descriptions. The microvilli were of the usual structure, possessing a distinct limiting membrane of trilaminar appearance, a central core of filaments extending into the terminal web region as rootlets, and a fuzzy glycocalyx covering the entire microvillar surface (Figs. 37 and 38).

At stage 42, the microvilli were often oriented at varying angles relative to the apical surface and appeared unevenly distributed along it (Fig. 37). Gradually all microvilli became oriented at 90° angles to the apical surface and assumed a more evenly distributed appearance (Fig. 38). Microvillar length increased from approximately 0.5 to 0.75 μ at stage 42 to 1.5 μ at stage 45, while

microvillar diameter (0.13 μ) did not change appreciably. At any stage there was considerable variability in microvillar length, and no differences in length could be detected in the different treatment groups.

Terminal Web Region

The area of apical cytoplasm adjacent to the brush border comprises the terminal web region (Figs. 24, 37, 38). In mature cells an extensive filamentous web, the terminal web, runs parallel to the apical surface at the level of the junctional complexes. In these cells filamentous organization was seen in the terminal web region at all stages, and while such organization became more extensive a prominant web was not evident by stage 45.

Apical pits and vesicles, possessing a membrane structure similar to that of the apical surface, were seen at all stages (Figs. 24, 37, 38). Also present in this region were ribosomes and agranular endoplasmic reticulum (Figs. 24, 38) and the microvillar rootlets. No changes were observed in this region which could be correlated with treatment.

Cytoplasm

The regions extending from the terminal web to the nucleus and from the nucleus to the basal membrane are referred to as apical and basal cytoplasm respectively.

The same organelles are found in both regions (with the exception of the Golgi complex).

A striking feature of the cytoplasm at stage 42 was the presence of many free ribosomes and polyribosomes (Figs. 23 to 25). At this time, granular or rough endoplasmic reticulum (RER) was visible as short tubular cisternae or vesicles (Figs. 23, 25), and agranular or smooth endoplasmic reticulum (SER) also appeared in the form of short tubules (Fig. 24). By stage 44 longer profiles of RER and SER were seen throughout the cytoplasm and ribosomes appeared mainly in groups or as polyribosomes (Figs. 29 to 35). Treatment with L-T_4 did not appear to affect the distribution of endoplasmic reticulum or ribosomes.

Mitochondria

Mitochondria were present throughout the cytoplasm, although they were typically more common apically. As the apical cytoplasm increased in area, more mitochondrial profiles appeared and individual mitochondria could be followed for longer lengths in sections (e.g. Fig. 33). The mitochondria were of typical structure with transversely oriented cristae and sometimes showing electron dense granules (Fig. 24). These organelles were similar in control and L-T_4 treated embryos.

Golgi Apparatus

In most sections of absorptive cells a portion of the Golgi apparatus could be seen occupying a supranuclear position. Typically several parallel Golgi saccules and many small vesicles appeared, and sometimes larger vacuoles were also present (Fig. 25). At stages 42 and 43 the Golgi apparatus often showed several saccules and vesicles, and sometimes the saccules were distended (Figs. 23, 26, 27, 28). By stage 45 the Golgi region showed several larger vesicles (Figs. 32, 33), however the most prominent Golgi at both stages 44 and 45 was exhibited by embryos which had been treated with L-T_4, 48 and 72 hours earlier respectively (Figs. 31, 34). These cells possessed many vesicles and vacuoles and greatly distended saccules.

Lysosomes and Related Bodies

Lysosomes and related bodies were present in variable numbers in the apical and basal cytoplasm. Frequently seen at all stages were multivesicular bodies, usually located in the apical cytoplasm (Figs. 23, 25a, 33). Small rounded electron dense bodies of approximately 0.5 μ diameter were also seen at all stages (Fig. 24a). These resembled dense bodies (primary lysosomes) by virtue of their electron dense contents and limiting membranes, however peroxisomes may appear similar and without the use of histochemical methods they may be

identified incorrectly. Secondary lysosomes with non-uniform contents were usually small and appeared infrequently in normal and D-T_4 treated cells at all stages (Figs. 23, 23a). Secondary lysosomes were more prominent in stage 44 and 45 epithelial cells from embryos which had been treated with L-T_4 48 and 72 hours previously (Figs. 31, 34, 35). These cells exhibited secondary lysosomes of variable shape and contents as well as small myelinated areas.

Glycogen

At stage 42 large deposits of particulate material resembling glycogen were frequently observed in the apical and basal cytoplasm (Figs. 23, 25). Often the deposits were 2 μ or more in diameter, as well small deposits and individual particles were scattered throughout the cytoplasm. Glycogen particles varied in size from 150 to 300 Å, while ribosomes were 170 to 230 Å in diameter.

The appearance of the glycogen deposits varied from extremely electron dense particulate (Fig. 25) to slightly electron dense and flocculent or less obviously particulate (Fig. 26a). At stages 42 and 43 both types of glycogen were seen. Later most of the glycogen deposits were small, and slightly electron dense, so that at low magnification they appeared as 'empty spaces' in the cytoplasm (Fig. 32). By stage 44 little glycogen was seen

in the absorptive cells of L-T_4 treated embryos (Fig. 31), while small deposits persisted in other embryos (Fig. 30). Stage 45 embryos which had been treated with L-T_4 48 hours previously still possessed glycogen deposits.

Lipid Droplets

Lipid droplets varied in size from the large (up to 3 μ in diameter) inclusions visible with the light microscope, to smaller droplets of less than 1 μ (Fig. 23a, 25, 27, 32). The droplets varied in electron density, although droplets of greater electron density appeared at the earlier stages. As well the droplets sometimes exhibited regions of varying electron density (Fig. 25). Often the droplets appeared to be surrounded by a limiting membrane (not a distinct unit membrane) and glycogen and myelinated areas were often associated with the perimeter (Fig. 25). Lipid droplets which persisted at stage 45 were generally slightly electron dense and lacked a limiting membrane (Figs. 32, 32a). In embryos treated with L-T_4, lipid droplets were less evident at stages 44 and 45 than in D-T_4 treated or normal embryos.

Nucleus

The large nucleus (approximately 3 μ in diameter and 6 μ in length) occupied much of the cell's area at stage 42. Generally the nuclei were rounded, but sometimes

they formed irregular projections (Fig. 23). Initially dispersed chromatin and one or more large nucleoli occupied the central area, while small amounts of condensed chromatin lined the nuclear membrane (Fig. 23). By stage 45 greater amounts of condensed chromatin were visible throughout the nucleus (Fig. 32). The double nuclear membrane was of typical structure (Fig. 24a). The appearance of the nucleus did not differ in L-T_4 treated and control groups.

Microfilaments and Microtubules

Microfilaments were often seen in the cytoplasm, particularly adjacent to basal and lateral membranes (Fig. 23a). Occasionally microtubules appeared in the cytoplasm, and often centrioles were seen in the apical regions of the cells (Figs. 24, 34). Neither of these components was observed to change with treatment.

b. Goblet Cells

Goblet cells observed in the duodenal epithelium of stage 42 to 45 embryos were usually in the intermediate state of development (Freeman, 1966). These cells were characterized by the presence of many mucous droplets in their apical cytoplasm and prominant Golgi complexes and endoplasmic reticulum (Figs. 30, 36). Such cells had not yet assumed the typical goblet cell shape, characterized by a large mucous filled theca and an electron

dense foot filled with compacted organelles. The mucous droplets varied in electron density and were fibrillar or flocculent in appearance. They usually showed limiting membranes and were sometimes coalesced. Glycogen and lipid droplets appeared in these cells as in the absorptive cells. Fully mature goblet cells were not observed electron microscopically, although some cells with prominent thecas had been observed using light microscopy. No changes in these cells were observed with L-T_4 treatment.

B Experiments Performed *In Vitro*

1. Light Microscopy

Duodenal explants from stage 42 embryos often showed changes in villus architecture following 48 hours of culture *in vitro*. Shortening and fusion of villi were observed, but often villi retained resemblance to their intact state (Fig. 39). The shape of the absorptive cells ranged from low columnar to elongated (Figs. 40 to 46). Cellular dimensions were similar to those of normal stage 42 to 45 cells, and the elongated cells were so named for their narrow basal regions which had the appearance of elongated processes (Fig. 41), not for an actual increase in cell length. Lateral cytoplasmic projections could be seen crossing the large intercellular spaces between adjacent elongated cells. Such cells often appeared to be normal in the form of their apical cytoplasm, but narrowed

from the level of the nucleus (Fig. 41). Nuclear and cytoplasmic staining were particularly intense in the elongated cells.

Cells which were normal in form, i.e. resembled the intact state often possessed lipid droplets (Figs. 40, 46). The cytoplasm of these cells appeared more granular or uneven than that of intact cells on staining. Many goblet cells with prominent mucous-filled thecas were seen in all cultures (Fig. 46).

2. Electron Microscopy

a. Absorptive Cells

The ultrastructure of duodenal absorptive cells which had been cultured *in vitro* for 48 hours was variable. Their cytoplasm often resembled the normal uncultured situation (Figs. 47, 49, 52), but just as often exhibited an appearance here described as condensed (Figs. 48, 50). The most condensed cells were those which appeared elongated with the light microscope. Many cells were intermediate in appearance between the normal and highly condensed state (Figs. 51, 53).

While in all groups it was attempted to obtain areas of 'normal' appearance to facilitate comparison, in some groups the best areas which could be obtained were highly altered in appearance. As these changes did not show any correlation to treatment they are assumed to

result from the culture and/or fixation procedures.

Absorptive Cells Showing Normal Appearance

Absorptive cells showing normal cytoplasmic appearance were often equivalent to about stage 43, (Fig. 49). However the amount of Golgi (Fig. 47) and relative amounts of ribosomes and endoplasmic reticulum varied, so that some cells were of more mature appearance than others. All of the organelles and inclusions described in the cells from the experiments performed *in vivo* were seen although centrioles seldom appeared. Generally, lipid droplets (Fig. 49a) and pale-staining glycogen were as plentiful as at stage 44 or 45. Secondary lysosomes and dense bodies were seen often but multivesicular bodies appeared infrequently.

Absorptive Cells Showing Condensed Appearance

Cells which showed cytoplasmic condensation (Figs. 48, 50, 51, 53) possessed large clusters of ribosomes, between which the cytoplasmic matrix appeared fibrillar and electron dense. Often RER and SER were not visible but occasionally extensive membranous structures of uncertain origin were seen (Fig. 50). Mitochondria in these condensed cells were often globular in shape with altered cristae and prominant granules (Fig. 51). Sometimes the mitochondria appeared coalasced, and might be

more correctly described as forming a mitochondrial mass (Fig. 48). Few apical pits and vesicles or multivesicular bodies were seen, and the Golgi complex was usually small. Some lysosomes (mainly secondary) were seen, and occasionally large areas of lysosomal activity (possibly autosomal) appeared. Glycogen (Fig. 48) and lipid droplets (Fig. 53) appeared mainly in cells which showed moderate condensation.

In cells showing cytoplasmic condensation and elongated or altered form, the cytoplasmic area was apparently reduced. Such cells might appear quite normal in the form of their brush border and terminal web regions, yet below the terminal web or apical cytoplasm showed reduced cytoplasm (Fig. 50). In these areas of reduced cytoplasmic area the main body of the cell's cytoplasm showed extreme condensation and packing of the organelles. Laterally extensive interdigitation of cytoplasmic processes of adjacent cells was seen across the enlarged intercellular spaces (Fig. 50). Sometimes the plane of cellular orientation appeared to have altered, giving the cells unusual shapes (Fig. 53).

In cells showing extreme condensation the nuclei usually showed more condensed chromatin than in non-condensed cells. Sometimes nuclear size was reduced as well, and in very extreme cases the nuclei became a highly compacted ball of condensed chromatin (Fig. 50).

Brush Border - Normal and Condensed Absorptive Cells

The appearance of the microvilli was quite normal whether or not the cells showed condensation (Figs. 54 to 60). In condensed cells the increased electron density of the cytoplasm made it difficult to discern the central cores and rootlets (Fig. 54). The angles of the microvilli to the apical surface varied, as did their distribution, length (mean approximately 0.7 μ, 0.9 μ) and diameter (mean 0.13 μ). Microvillar length was often similar to stage 42, while arrangement and distribution resembled stage 44.

The glycocalyx formed a consistantly thick layer in cells cultured with 14 ng L-T_4/ml of medium, with or without BSA (Figs. 56, 57). In other groups the glycocalyx varied from a fine fuzzy covering on the apical membrane (Figs. 54, 58) to a definite layer (Fig. 55). No other changes in the brush border could be consistantly related to any one treatment medium.

Some areas were seen which appeared to be sparsely covered with microvilli (Fig. 61). In such areas the microvilli were short (0.3 μ or less) thus might appear less plentiful when sectioned. However, the apical membrane in such regions could be followed for distances of up to 1 μ without interruption, indicating that microvilli may have been lacking. Extremely long microvilli were not observed, neither was

blebbing of the microvilli to form vesicles.

b Goblet Cells

In duodenal explants which had been cultured *in vitro* for 48 hours, goblet cells of mature appearance were seen (Fig. 62). These possessed a large mucous filled theca (up to 3 μ in diameter and 6 μ in length), and an electron dense, condensed foot region. The mucous droplets varied in size and electron density and sometimes evidence of their extrusion into the lumen (Fig. 62a) was seen. The foot region contained a Golgi complex with extended saccules and extensive RER with electron dense contents. Also present in this region were longitudinally oriented, electron dense mitochondria and a condensed nucleus. Condensed absorptive cells often resembled the goblet cell foot making identification of a foot sectioned without its theca difficult. Well developed goblet cells were seen in all groups, and their structure was not observed to differ with treatment.

LIGHT AND ELECTRON MICROGRAPHS

Abbreviations Used In Micrographs

AP	Apical Pit	LD	Lipid Droplet
AV	Apical Vesicle	M	Mitochondrion
BL	Basal Lamina	MA	Tight Junction
C	Centriole	MT	Microtubule
CC	Condensed Chromatin	MY	Myelination
DB	Dense Body	MU	Mucous
DC	Dispersed Chromatin	MV	Microvillus
F	Microfilament	N	Nucleus
G	Golgi Complex	NU	Nucleolus
GC	Goblet Cell	R	Ribosome
GL	Glycogen	RER	Granular Endoplasmic Reticulum
GS	Golgi Saccule	SER	Agranular Endoplasmic Reticulum
GV	Golgi Vesicle		
I	Interdigitation	TJ	Junctional Complex
ICS	Intercellular Space	TW	Terminal Web
L	Lysosome	VAC	Vacuole

Figures 8 to 22. Light micrographs of villi and duodenal epithelium from experiments performed in vitro. All sections were from material embedded in Araldite 502, and stained with toluidine blue. These figures illustrate the increasingly columnar appearance of the epithelium with increased stage. Figures 8, 10, 14, 18 x 640. Other Figures x 2520.

Fig. 8. Stage 42 normal. Apical portion of villus. showing cuboidal to low columnar shape of epithelial cells.

Fig. 9. Stage 42 normal. The absorptive epithelial cells contain large nuclei with prominant nucleoli. Several large rounded inclusions (lipid droplets) are seen in the basal cytoplasm.

Fig. 10. Stage 43 normal. Note slightly more columnar appearance of epithelium compared to stage 42.

Fig. 11. Stage 43 normal.

Fig. 12. Stage 43 D-T_4 treated (24 hr).

Fig. 13. Stage 43 D-T_4 treated (24 hr).

Fig. 14. Stage 44 normal. Note increased apical cytoplasmic area of epithelial cells.

Fig. 15. Stage 44 normal. The brush border is now distinct (arrow).

Fig. 16. Stage 44 D-T_4 treated (48 hr).

Fig. 17. Stage 44 L-T_4 treated (48 hr).

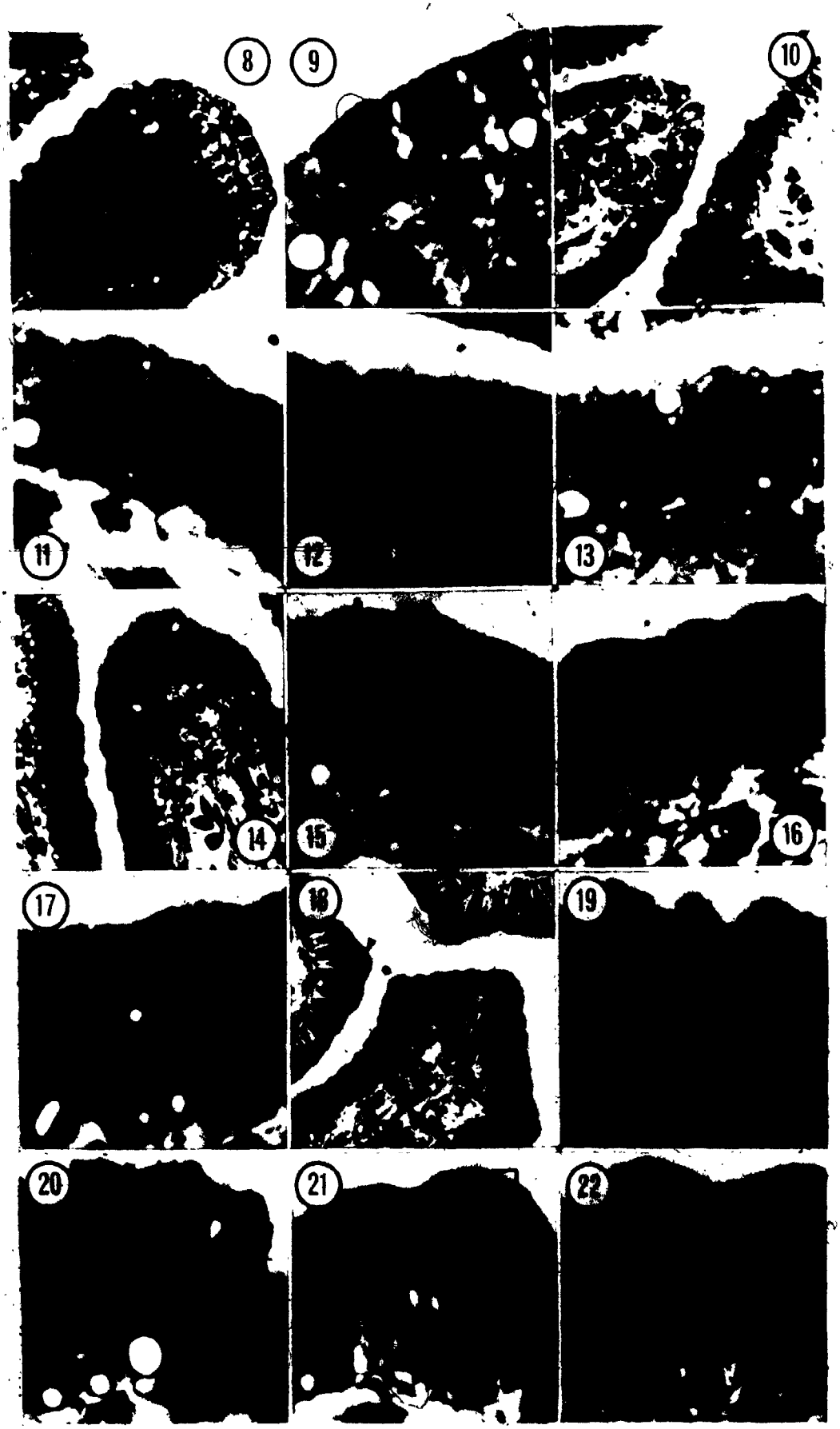
Fig. 18. Stage 45 normal. Note the lack of lipid droplets compared to earlier stages and pale-staining areas above the nuclei of many cells.

Fig. 19. Stage 45 normal. A goblet cell is visible (arrow) due to the pale-staining mucous in its apical cytoplasm,

Fig. 20. Stage 45 D-T_4 treated (72 hr).

Fig. 21. Stage 45 L-T_4 treated (72 hr). A goblet cell is visible at arrow.

Fig. 22. Stage 45 L-T_4 treated (48 hr).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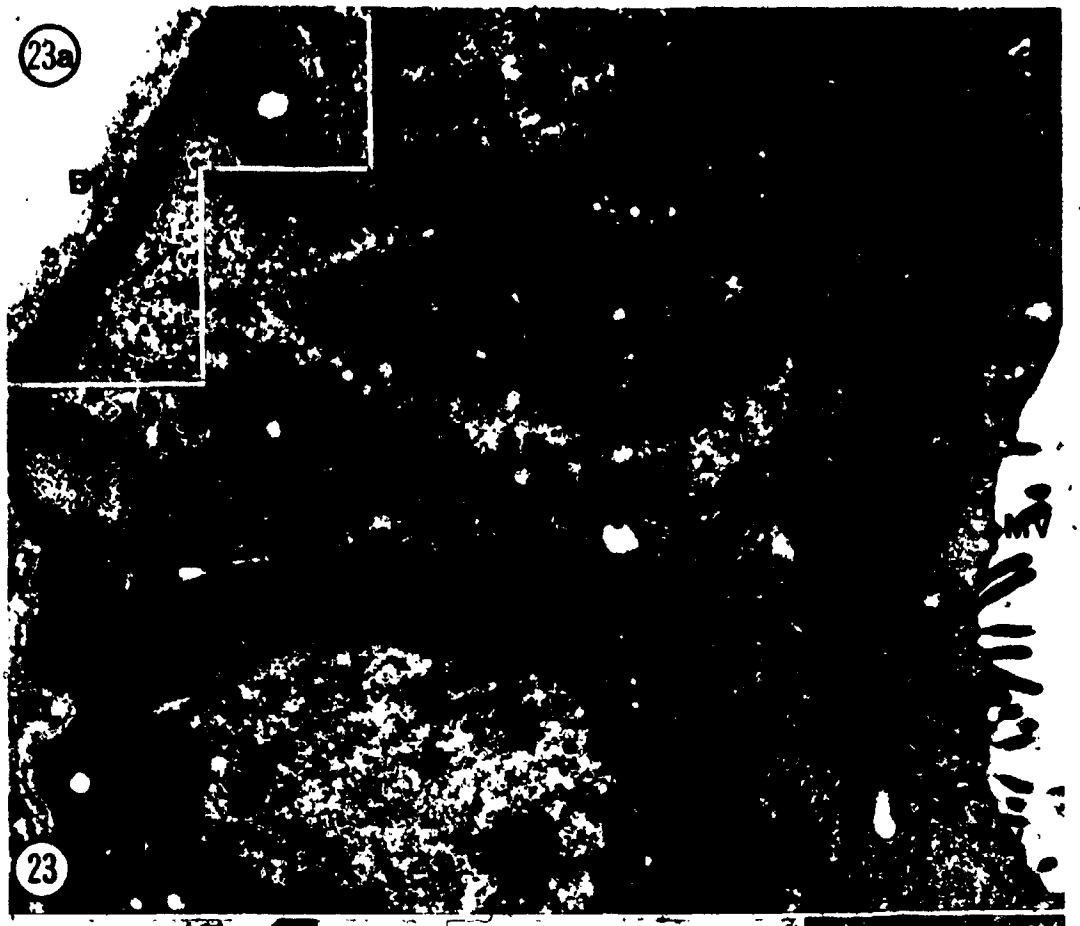
Figures 23 to 38. Electron micrographs of duodenal epithelium from experiments performed *in vivo*. Material illustrated was fixed with half strength Karnovsky fixative, post-fixed with OsO_4 in sodium cacodylate or Millonig's buffer, embedded in Araldite 502, and stained with uranyl acetate and lead citrate. Material post-fixed using Millonig's buffer is designated by 'MB' in legends.

Fig. 23. Longitudinal section through columnar absorptive cells of duodenal epithelium of normal stage 42 embryo. The major regions of the absorptive cells are seen: the brush border composed of microvilli (MV), the terminal web (TW), and the apical and basal cytoplasm separated by the nucleus (N). The nucleus contains one or more prominant nucleoli (NU) and is filled mainly with dispersed chromatin (DC), condensed chromatin (CC) lining the membrane. The cytoplasm contains mitochondria (M), lysosomes (L), Golgi (G), glycogen (GL), and lipid droplets (LD). The lateral surfaces of adjacent cells are held in close apposition by juxta-luminal junctional complexes (TJ), and tight junctions (macula adherens, MA). Interdigitations (I) occur on their lateral surfaces, and basally a thin basal lamina (BL) underlies the epithelium. MB x 12000.

Fig. 23a. Basal surface (stage 42 normal) showing filamentous basal lamina. Microfilaments (F) are seen in the basal cytoplasm adjacent to the cell membrane. A small secondary lysosome with non-uniform contents is seen. x 42000.

Fig. 24. Apical cytoplasm of stage 42 normal absorptive cells. Several apical pits (AP) and apical vesicles (AV) are seen. Many free ribosomes (R) and microfilaments are seen. Several rounded electron dense bodies (DB) are seen associated with agranunular endoplasmic reticulum (SER). Microtubules (MT) and a centriole (C) appear. MB. x 24000.

Fig. 24a. A dense body (primary lysosome) with electron dense contents and distinct limiting membrane is seen. Also visible is a portion of a lipid droplet which appears to be bounded by a less distinct type of limiting membranous structure. The double nature of the nuclear membrane is visible, the outer portion being ribosome studded (arrow). MB. x 30000.


23a
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24
24a

Fig. 25. Apical cytoplasm of stage 42 normal absorptive cells showing prominent glycogen deposits. Note the range in size of glycogen particles. The contents of the lipid droplets varies in electron density and glycogen is often located at their perimeters. Several short profiles of granular endoplasmic reticulum (RER) and SER, and many free ribosomes are seen. The Golgi complex shows saccules (GS), small vasicles (GV), and larger vacuoles (VAC). MB. x 30000.

Fig. 25a. Multivesicular body. Its single limiting membrane encloses many small vesicles, each with its own limiting membrane (arrows). Several other small vesicles are seen in close proximity to the multivesicular body. x 48000.

Fig. 26. Longitudinal section through stage 43 normal absorptive cells. Appearance is similar to stage 42 (Fig. 23). Ribosomes often appear in small groups or rosettes (circles). A myelinated area, possibly a remnant of secondary lysosomal activity (MY) and intercellular spaces (ICS) are seen. Glycogen is less electron dense than the surrounding cytoplasm. MB. x 12000.

Fig. 26a. Pale staining glycogen from same block as Fig. 26. MB. x 30000.

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25c
VAC

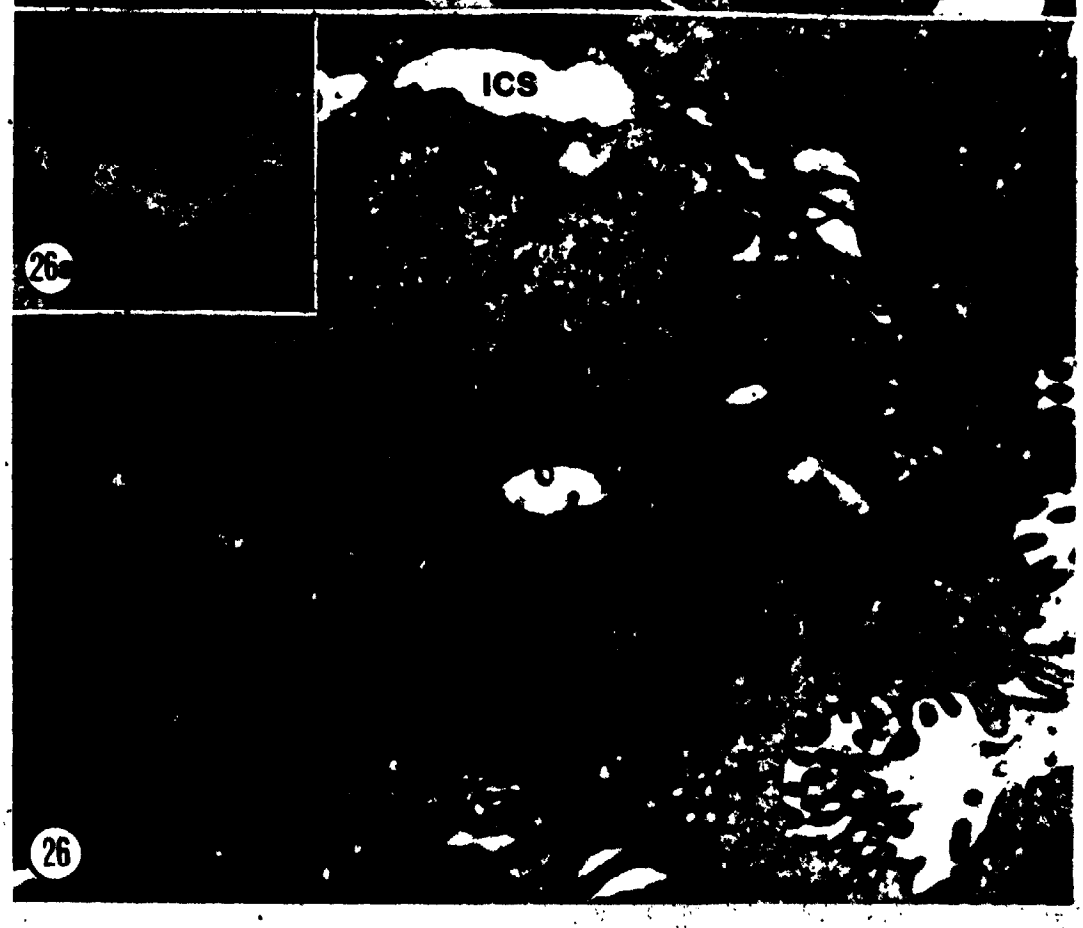
ICS
26c
26

Fig. 27. Longitudinal section through columnar absorptive cells of stage 43 duodenum, 24 hr after treatment with D-T_4. Cellular appearance is similar to stage 43 normal (Fig. 26). MB. x 11000.

Fig. 28. Longitudinal section through columnar absorptive cells of stage 43 duodenum, 24 hr after treatment with L-T_4. The cells appear similar to stage 43 normal and D-T_4 treated duodenum (Figs. 26 and 27). Note the presence of long profiles of RER and several secondary lysosomes. Glycogen particles appear strongly electron dense. x 8800.

27

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Fig. 29. Longitudinal section of stage 44 normal columnar absorptive cells. The trends seen at stage 43 of increasing ribosomal rosettes and RER are continued. Many apical pits and vesicles are seen. The brush border shows increased regularity in microvillar arrangement. x 6600.

Fig. 30. Longitudinal section of stage 44 duodenal epithelium 48 hr after treatment with D-T_4. Absorptive cells appear slightly more mature than stage 44 normal (Fig. 29) due to larger apical cytoplasm, lower position of nuclei, and more profiles of mitochondria and RER. A pre-goblet cell (GC) is seen containing many Golgi vesicles and large mucous droplets (MU) of flocculent appearance. x 8800.

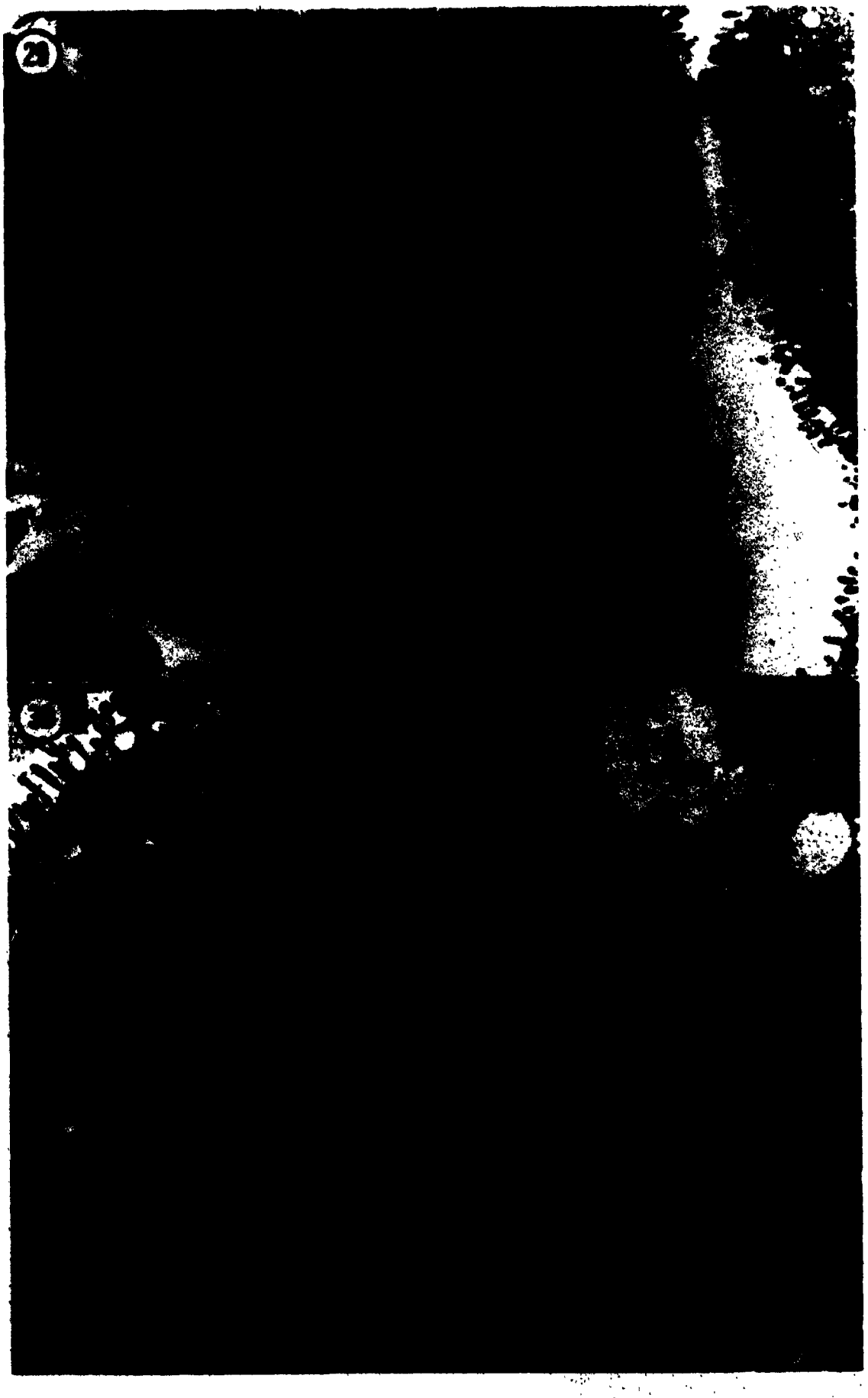
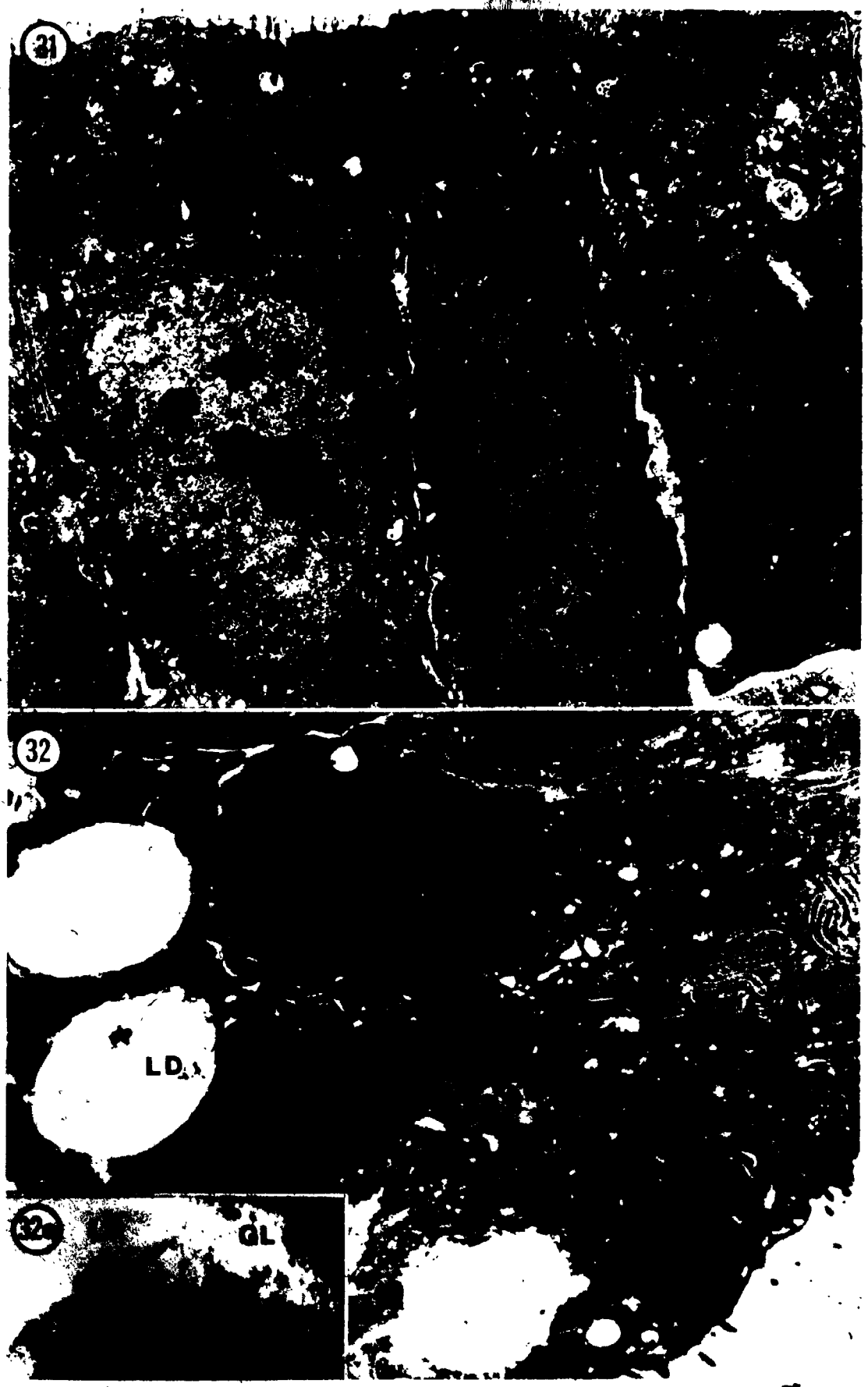


Fig. 31. Longitudinal section of absorptive cells of stage 44 duodenum 48 hr after treatment with L-T_4. Compared to stage 44 D-T_4 treated (Fig. 30) the Golgi apparatus is more prominent, and secondary lysosomes and small areas of myelination seem to appear more frequently (arrows). x 8800.

Fig. 32. Oblique section of stage 45 normal columnar absorptive cells. The presence of many glycogen 'spaces' of slight electron density, Golgi and apical vesicles gives the cytoplasm a foamy appearance. Several lipid droplets of slight electron density are seen. Note the presence of many cisternae of RER containing electron dense material (arrows). Interdigitation of the lateral membranes is more extensive than in earlier stages. The nuclei show increased amounts of condensed chromatin. x 8800.

Fig. 32a. Enlargement of area adjacent to a lipid droplet (stage 45 normal). The contents of the lipid droplet appear continuous with adjacent spaces containing slightly electron dense material resembling glycogen particles. x 54000.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32
LD
32c
GL

Fig. 33. Longitudinal section of columnar absorptive cells stage 45 duodenum 72 hr after treatment with D-T_4. Fewer glycogen spaces are seen than in previous figure, and the Golgi apparatus appears similar to slightly more extensive. Many polyribosomes and extensive RER appear. The mitochondria show unusually intense electron density. x 8800.

Fig. 34. Longitudinal section of columnar absorptive cells of stage 45 duodenum 72 hr after treatment with L-T_4. The Golgi apparatus is more extensive and secondary lysosomes (arrows) appear more frequently than in stage 45 normal and D-T_4 treated embryos (Figs. 32 and 33). Few glycogen spaces, many polyribosomes, and much RER appear. The brush border and terminal web regions are highly organized. Cells close to the villus tip are more elongated and show large intercellular spaces . x 6600.

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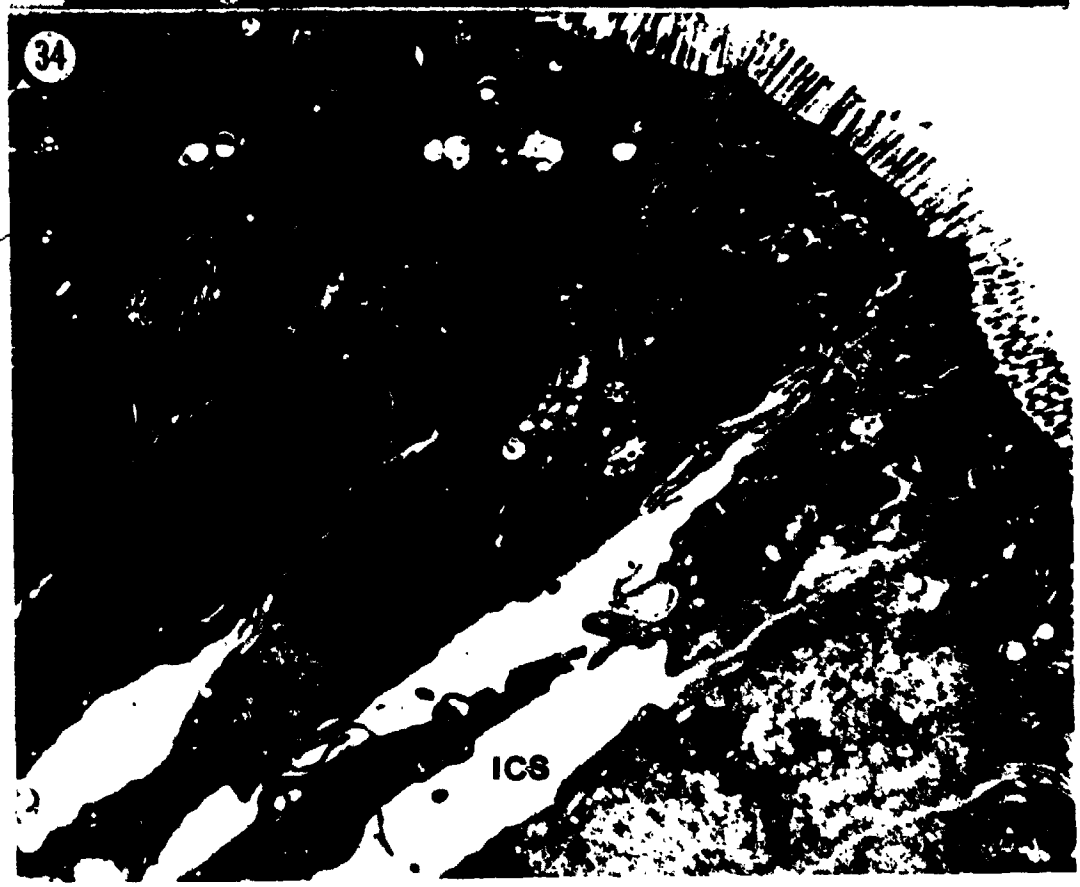
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ICS

Fig. 35. Longitudinal section of columnar absorptive cells of stage 45 duodenum 48 hr after treatment with L-T_4. RER and polyribosomes appear frequently. Several areas of slightly electron dense glycogen are seen. Some secondary lysosomes appear. The Golgi apparatus appears similar to that of stage 44, 48 hr following D-T_4 treatment (Fig. 30). x 6600.

Fig. 36. Longitudinal section of a pre-goblet cell (stage 42 normal). Note the similarity of the cytoplasm to that of adjacent absorptive cells. A Golgi apparatus with distended saccules and several vesicles appears. Mucous droplets of varying electron density are seen in the apical cytoplasm. At lower right a portion of the goblet cells brush border is seen. x 6600.

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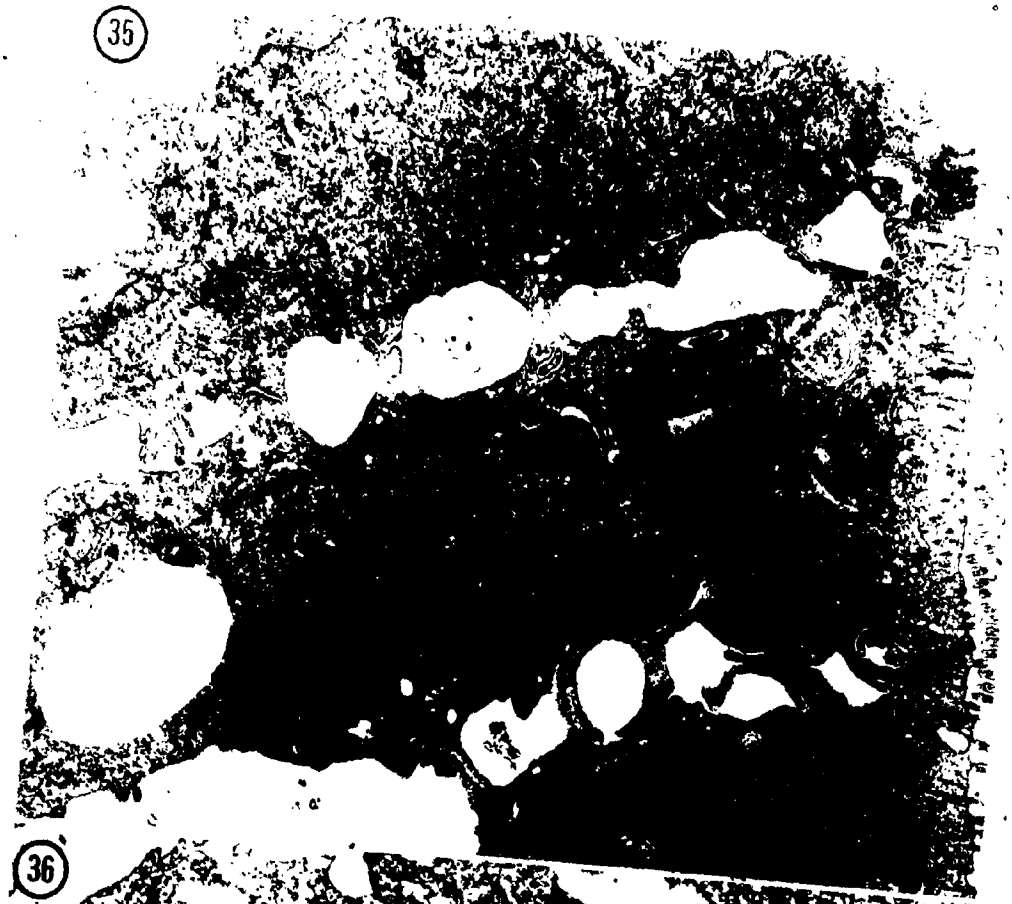

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Fig. 37. Longitudinal section through microvilli of the brush border of stage 42 (normal) absorptive cell. The microvilli project at varying angles from the apical surface and seem to vary in height. The plasma membrane appears trilaminar and its outer surface is covered by the filamentous glycocalyx. A central core of filaments is found in each microvillus (arrow) and extends into the terminal web region as rootlets (R). Filaments running parallel to the apical membrane are seen in the terminal web region. A portion of the juxta-luminal junctional complex appears in oblique section in the lower portion of the figure. An apical vesicle (AV) is seen in the terminal web region. x 60000.

Fig. 38. Longitudinal section through microvilli of the brush border of stage 45 absorptive cell (72 hr after treatment with D-T_4). The microvilli are similar in structure to those in preceding figure, but now form 90° angles with the apical surface. They are also taller and slightly narrower. An apical pit is seen. Note the continuity of its membrane with the apical surface (arrow). Several cisternae of SER appear. x 60000.

Figures 39 to 46. Light micrographs of duodena explanted at stage 42 and cultured *in vitro* for 48 hr using McCoy's 5A medium, to which T_4 and/or BSA were added as indicated. All material was embedded in Araldite 502 and stained with toluidine blue. Many absorptive cells show intense cytoplasmic and nuclear staining and often they are elongated or altered in form.

Fig. 39. Longitudinal section through villus cultured 48 hr with normal medium plus BSA. Villus architecture appears quite normal. (Compare with Figs. 8, 10, 14). x 640

Fig. 40. Absorptive epithelium cultured in normal medium without additives. Cell shape is somewhat elongated. Inclusions are difficult to distinguish from intercellular spaces. The brush border is evident. x 2500.

Fig. 41. Elongated absorptive cells cultured in normal medium plus BSA. Apical regions of cells appear quite normal. Nuclei appear compressed, and in the nuclear and basal regions the cytoplasm consists of processes between cells and an elongated cytoplasmic tail. x 2500.

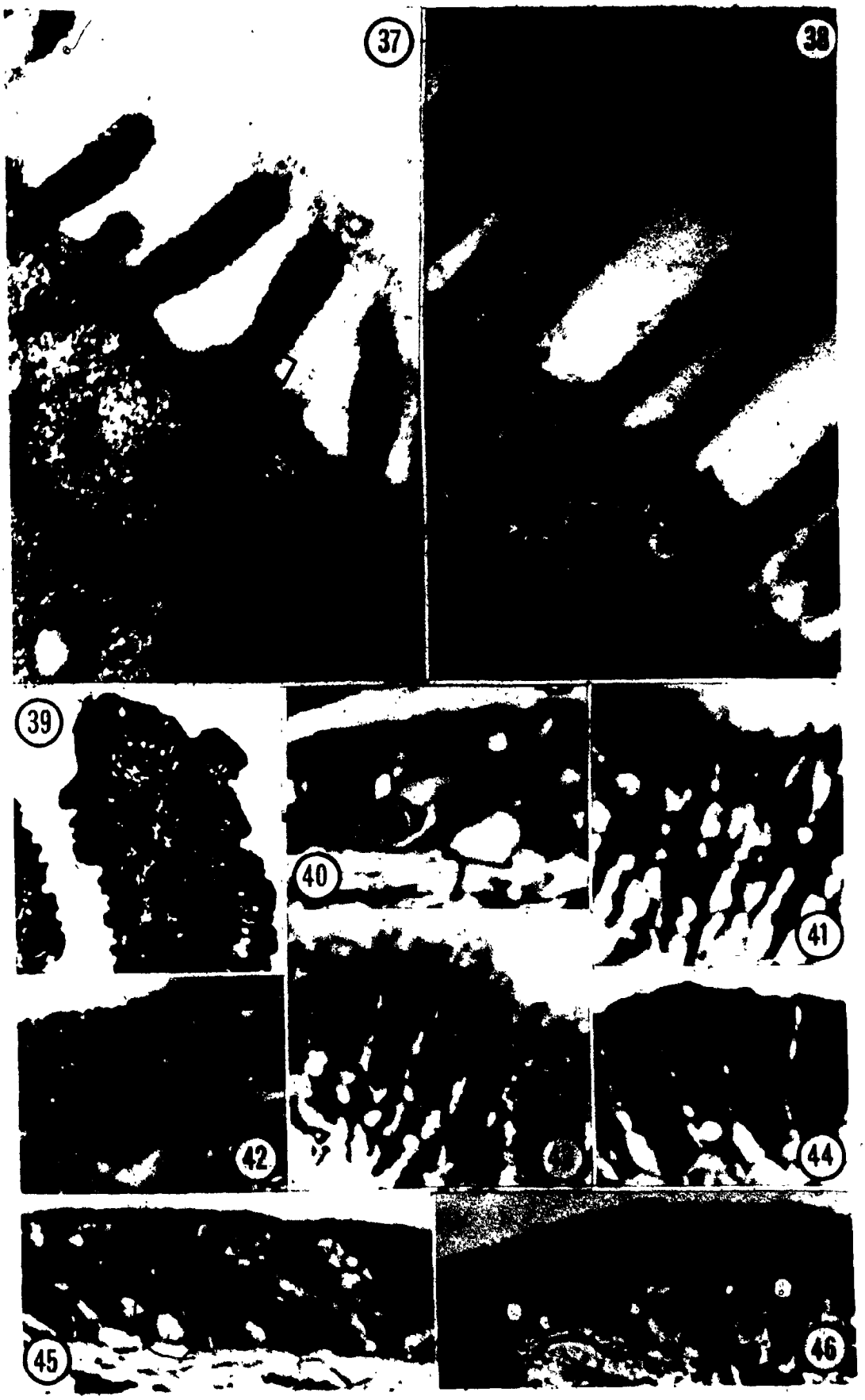
Fig. 42. Absorptive cells cultured with 14 ng L-T_4 /ml of medium. The nuclei stain intensely and the cytoplasm appears granular. Cells exhibit normal low columnar appearance. x 2500.

Fig. 43. Absorptive cells cultured with 14 ng L-T_4 /ml plus BSA in the media. The cells appear intermediate in form between normal and elongated appearance. Many pale bodies appear in the apical cytoplasm. A lightly staining area is visible above the cells' apical surface. x 2000.

Fig. 44. Absorptive cells cultured with D-T_4 and BSA in the medium. Intercellular spaces are enlarged but overall shape is normal. A large lipid droplet is seen. x 2500.

Fig. 45. Absorptive epithelium cultured with 14 ng L-T_4 / 100 ml of medium. Cellular orientation appears altered. x 2000.

Fig. 46. Absorptive epithelium cultured with 14 ng L-T_4 / 100 ml of medium plus BSA. The cells appear normal and the mucous of a goblet cell can be seen (arrow). x 1600.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Figures 47 to 62a. Electron micrographs of duodenal epithelium from experiments performed in vitro. All duodena were explanted at stage 42 and cultured 48 hr with McCoy's 5A medium containing T_4 and/or BSA as indicated. Material illustrated was fixed according to Buckley's procedure (1973), post-fixed with OsO_4 in sodium cacodylate buffer, embedded in Araldite 502, and stained with uranyl acetate and lead citrate.

Fig. 47. Longitudinal section of columnar absorptive cells cultured with normal (McCoy's 5A) medium. The cells resemble normal stage 42 uncultured absorptive cells (Fig. 23) in form and cytoplasmic appearance. Many free ribosomes appear in the cytoplasm and RER and SER are not extensive. Several apical vesicles and secondary lysosomes appear. A Golgi apparatus with many saccules is seen above the nucleus. The mitochondria appear electron dense and show some alteration in shape. Small amounts of slightly electron dense glycogen can be observed. x 18000.

Fig. 48. Oblique section through apical and nuclear regions of epithelium cultured with normal medium containing BSA. The absorptive cells have a 'condensed' appearance; their electron dense cytoplasm contains many large groups of ribosomes, and endoplasmic reticulum is not easily discerned. The Golgi complex consists mainly of saccules. A very large, abnormally shaped mitochondrion and bodies resembling lysosomes are seen. The brush border and terminal web regions are normal in form although their structural details cannot be discerned. Note the many small deposits of glycogen. Portions of two goblet cells appear in the figure. x 24000.

Fig. 49. Longitudinal section of absorptive cells cultured with medium containing D-T_4 and BSA. The cells are normal in form and appearance. Their cytoplasm contains many free ribosomes, short profiles of RER and SER, a small Golgi complex, small glycogen deposits, and mitochondria of normal appearance with prominent mitochondrial granules (arrow). The microvilli appear to be of variable length but are mainly at 90° to the apical surface; the terminal web appears normal and shows several apical pits and vesicles. The nuclei appear normal and show moderate amounts of condensed chromatin. x 24000.

Fig. 49a. Basal portion of absorptive cell cultured with medium containing D-T_4 and BSA. A portion of a lipid droplet which lacks a limiting membrane is at top of figure. Several small glycogen 'spaces' appear. The basal lamina appears slightly dispersed. x 30000.

49

Fig. 50. Longitudinal section of absorptive epithelial cells cultured with 14 ng L-T_4 /ml of medium. These cells show extreme cytoplasmic and nuclear condensation. From left to right in the figure, the nuclei show progressively more condensed chromatin and reduction in size. The mitochondria are of abnormal shape and have a coalesced appearance. Ribosomes appear as large groups. A large membranous structure (arrows), possibly derived from compressed interdigitations, is seen. The brush border terminal web regions appear normal in form and the glycocalyx is prominent. Below the terminal web the cytoplasm appears reduced in area, the intercellular spaces appear enlarged and extensive interdigitations are visible. x 12000.

Fig. 51. Oblique section of absorptive cells cultured with 14 ng L-T_4 /ml and BSA. Cytoplasmic condensation is not as great as in the previous figure. Ribosomes appear mainly in groups. Profiles of RER and SER, and a small Golgi complex are visible. The mitochondria are abnormal and areas of myelination (secondary lysosomes) are visible. x 18000.

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Fig. 52. Oblique section of absorptive cell cultured with 14 ng L-T_4 /100 ml of medium. The cytoplasm and organelles are normal in appearance although the cytoplasmic area appears reduced, possibly due to the plane of section. x 25600.

Fig. 53. Oblique section of absorptive epithelium cultured with 14 ng L-T_4 /100 ml and BSA. The cytoplasm and nuclei show condensation and a large lipid droplet appears. The cell at upper left contains several mucous droplets. x 18000.

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MY

Figures 54 to 61 illustrate the variations in appearance of the microvilli of the absorptive cells following 48 hours in culture. All magnifications x 60000.

Fig. 54. Normal (McCoy's 5A) medium + BSA. Brush border of a condensed cell shows microvilli of normal form although their internal structure and glycocalyx cannot be distinguished. Their 90° angle to the surface and frequent distribution resemble the stage 44 to 45 uncultured appearance (Fig. 38). Microvillar length is similar to stage 42 (Fig. 37). The diameter of these is smaller than usual.

Fig. 55. Normal medium. Microvilli of cell with normal cytoplasmic appearance.

Fig. 56. L-T_4, 14 ng/ml. Microvilli of condensed cell. The glycocalyx is very dense.

Fig. 57. L-T_4, 14 ng/ml + BSA. Several of the microvilli appear shortened. Absorptive cell was moderately condensed. The glycocalyx is prominant.

Fig. 58. L-T_4, 14 ng/100 ml. Microvilli of moderately condensed absorptive cell. The glycocalyx appears as a fuzzy coat on the membrane.

Fig. 59. L-T_4, 14 ng/ml + BSA. Microvilli of moderately condensed cell.

Fig. 60. D-T_4 + BSA. Microvilli of cell with normal cytoplasmic appearance.

Fig. 61. L-T_4, 14 ng/100 ml. An area of the apical surface of a moderately condensed cell is shown. The trilaminar appearance of the membrane can be traced for a long distance without interruption by microvilli projecting out of the plane of the section. The membrane's fuzzy coat is visible (arrow).

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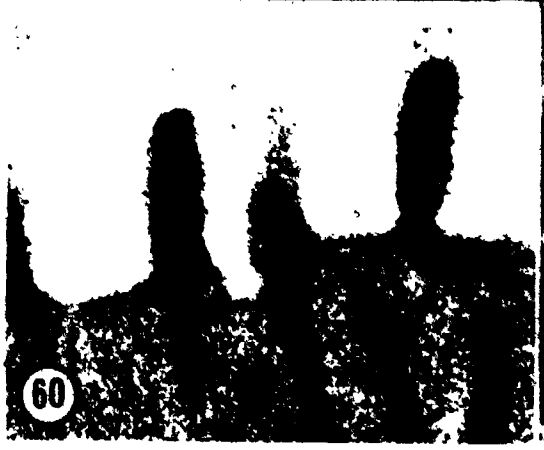
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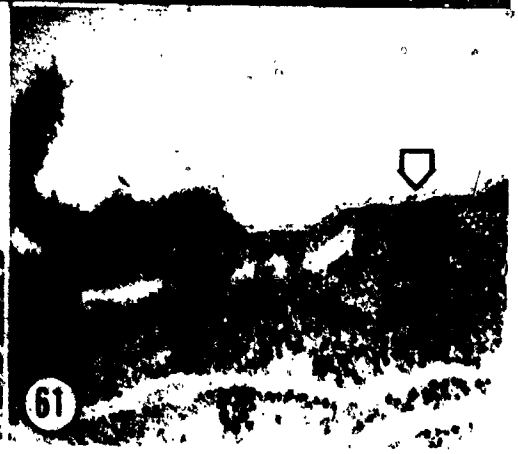
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Fig. 62. Oblique section of duodenal epithelium cultured with L-T_4 (14 ng/100 ml) and BSA. A goblet cell with a large mucous filled theca is seen in an area of condensed absorptive cells. The goblet cell's foot region contains many ribosomes and cisternae of RER which are filled with electron dense material. A portion of the Golgi apparatus is seen below the mucous. A large mitochondrion containing many electron dense granules appears in the foot region. Note the similar cytoplasmic electron densities of the goblet and absorptive cells. x 18000.

Fig. 62a. Mucous droplets adjacent to theca of a goblet cell (L-T_4 14 ng/100 ml + BSA). The droplets appear to be in the process of leaving the cytoplasm (exocytosis). The droplets appear to be located in a separate cell from that which possesses the theca at the bottom of the figure, possibly due to the angle of the section. x 60000.

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MU
62a

DISCUSSION

Experiments Performed *In Vivo*

Treatment Time

When embryos are treated *in ovo* as in this experiment, the developmental stage (Hamburger and Hamilton, 1951) at which an individual actually receives treatment can never be known with certainty. While three or more stages were found at each time examined, the stage forming the largest segment of the population increased by one with each 24 hour interval. Therefore, the stage at treatment could be estimated by subtracting the appropriate number of stages for each 24 hour period elapsed. The obvious drawback to this method is that it assumes that development proceeds uniformly during this period in all embryos, which is unlikely. As well, stage 45 can last up to 48 hours (ibid.), but it could not be determined how much time the embryo had spent in that stage, therefore it was assumed to be 24 hours or less for estimation of treatment time.

Stage 45

For analysis of the distribution of developmental stages stage 45 was subdivided into two groups. Embryos

which had begun to pip their shells were grouped separately from those which had not, as the former might be considered more advanced due to the presence of a visible sign of hatching. However, pipping and hatching times vary considerably (Freeman and Vince, 1974), so that separation of the biochemical data in a similar fashion would not necessarily have increased accuracy. Examination of the alkaline phosphatase and protein data indeed did not reveal any differences in the two sub-groups, nor was variation any less. Therefore, for the time encompassed by this experiment, use of the developmental stages as recognized by Hamburger and Hamilton (1951) seemed adequate, although the limitations imposed by the length of stage 45 must be recognized.

Effects of Thyroxine Treatment on Stage

Thyroxine treatment has been observed to produce early hatching (Vidal, 1953; Balaban and Hill, 1971), and thiourea treatment to delay hatching (Grossowicz, 1946; Adams and Bull, 1949; Balaban and Hill, 1971). While a larger proportion of the embryos in the L-T_4 treated group had begun to pip their shells by 72 hours, the present experiment was not continued long enough to determine whether hatching time would be earlier than normal. It did not appear that earlier overall development was stimulated by L-T_4 as the stage distributions were similar with all times and treatments.

Effects of Thyroxine on Yolk Sac Retraction

In thiourea-treated embryos, retraction of the yolk sac into the abdominal cavity is greatly delayed (Grossowicz, 1946; Adams and Bull, 1949; Balaban and Hill, 1971). Moog (1961b) found that T_4 treatment accelerated yolk sac retraction at 19 days, and Vidal (1953) found that T_4 caused more rapid retraction without affecting its time of onset. Recently Wishart *et al.*, (1977) have induced retraction as early as stage 43 using T_4. In the present study, yolk sac retraction was observed to begin early with L-T_4 treatment; half of the stage 44 L-T_4 treated embryos had begun retracting their yolk sacs, while no other group showed retraction before stage 45.

Mortality

In the experiments performed *in vivo*, a dosage equivalent to 5 μg of hormone was used (5.7 μg T_4 - pentahydrate salt). Similar dosages have been used to treat chicken embryos by several investigators (Grossowicz, 1946; Vidal, 1953; Adams, 1958). Vidal found 36% mortality following 3 treatments of 5 μg L-T_4 each, at 7, 14, and 17 days, while Konigsberg (1958) found that a total dosage of more than 2.3 μg over days 12 to 18 was in the thyrotoxic range.

Twenty-four hours after treatment, all groups showed fairly similar mortalities (16 to 24%). At 48 hours,

mortality (determined from the time of treatment) was much larger in the L-T_4 treated than in all other windowed groups, but similar to that of the normal (unwindowed) group. At seventy-two hours the total losses were about 50% in most control groups (except saline-treated: 33%), and 75% in the L-T_4 treated group. Therefore mortality appears to have been elevated 48 and 72 hours after L-T_4 treatment.

The mortalities found 48 hours after treatment show several discrepencies compared to the other times. At 48 hours survival was better in all the windowed groups (except L-T_4 treated) than at 24 hours. This was possible as mortality was determined only among the embryos to be killed at a particular time to avoid unnecessary disturbance of the other groups. However it indicates that normal mortality may be quite variable.

The much larger loss shown by the normal group at 48 hours compared to the windowed controls suggests that windowing might eliminate embryos which would normally die at this time, but still one would expect to find equally good survival of windowed embryos at 24 hours. Mortality in the normal group may have been elevated by failure to eliminate all dead embryos initially through candling. However, checks of candled eggs revealed that correct identification of dead embryos was made with greater than 90% accuracy, therefore would not account for large increases in apparent mortality in this group.

The mortalities shown by the control groups over the course of the experiment probably represent the peak in embryonic mortality which is commonly reached between 18 to 20 days (Byerly, 1931); the magnitude of this peak varies in different strains (Landauer, 1951). Landauer (1951) noted that the normal peaks in embryonic mortality seem related to important changes in metabolic activity at these times.

Whether the increased mortality which was found with L-T_4 treatment indicates that the dosage used was pharmacological cannot be determined satisfactorily from this data. As yolk sac retraction and pipping were advanced in these birds, they may also have been demonstrating hatching mortality earlier than the control groups at 72 hours. Peaks at these two times are often related in magnitude (ibid.).

Variation in Alkaline Phosphatase Specific Activities

In the experiments performed *in vivo*, large variations in alkaline phosphatase specific activity were observed within treatment groups. This tendency, indicated by increasing standard errors of the mean, increased with increasing specific activity and therefore with advancing stage and time. In the experiments performed *in vitro*, specific activities did not exceed those normally observed at stage 43, and showed similarly small variations.

The biochemical procedures were tested (as described in Appendix V) to determine whether they could be causing variation. In these tests the maximum variation was 4.4% (44 units per 1000), which could account for a substantial portion of the variability at the lowest specific activities but not at the highest.

Previous authors have found large variation in the duodenal alkaline phosphatase of chicken embryos (Moog and Richardson, 1955; Bellware and Betz, 1970; Hart and Betz, 1972b), therefore it seems that individual variation is inherent in this system. However, this introduces the problem that values must be very different at stage 45 (or 72 hr) for significant differences to be found. Duncan's New Multiple Range analysis (1955, 1957; see Kirk, 1968) takes unequal variance into account, while allowing the simultaneous comparison of many groups.

Bellware (1969) questioned the use of specific activity (μg phenolphthalein/hr/mg protein) as an expression of phosphatase in duodenal homogenates. He felt that it would be affected by variations in protein content, and that albumen adsorbed onto the luminal surface might raise the protein values; therefore he expressed phosphatase relative to dry duodenal weight. Hart (1971) found the patterns of alkaline phosphatase development to be very similar using either measure. As both dry weight and total protein content are derived in large part from the duodenal muscle mass, while alkaline

phosphatase is located mainly in the brush border of the absorptive epithelium (Gomori, 1941; Moog, 1950; Holt and Miller, 1962; Overton et al., 1965; Hugon and Borgers, 1966), the choice appears to be arbitrary.

Effects of Thyroxine Treatment on Duodenal Alkaline Phosphatase

The increase in duodenal alkaline phosphatase specific activity seen from stages 41 to 45 (or days 16 to 19) was similar in pattern and time course to the observations of previous authors (Moog, 1950, 1961b; Moog and Richardson, 1955; Bellware and Betz, 1970; Hart and Betz, 1972a). The onset of the steepest phase of increase was previously found to occur at day 18 (Moog, 1950, 1961b; Moog and Richardson, 1955) or 19 (Bellware and Betz, 1970; Hart and Betz, 1972a), in unstaged embryos. Here the phase of steepest increase was observed to occur between stages 44 and 45, corresponding to approximately 18.5 to 19.5 days of incubation.

Alkaline phosphatase accumulation was observed to begin earlier in groups treated with L-T_4. Twenty-four hours after treatment, phosphatase activity was elevated slightly in such embryos (stage 43). At 48 hours, the duodenal alkaline phosphatase activity of stage 44 L-T_4 treated embryos was significantly greater than in any other stage 44 group, and was comparable to that of stage 45 controls, 24 hours later. By seventy-two hours, the

phosphatase activity of L-T_4 treated stage 45 embryos was further elevated, although no longer significantly due to the large variation in specific activity in all groups. This was greatest in the windowed group, and was probably more prominent due to this group's smaller sample size. However, even if this group was omitted from the statistical comparison, significant differences did not appear between the stage 45 L-T_4 treated (largest) and saline-treated (smallest) specific activities. Therefore L-T_4 treatment apparently stimulated phosphatase accumulation to occur 24 hours earlier than normal.

Stage 43 and 45 embryos also showed elevated alkaline phosphatase specific activity 48 hours after treatment with L-T_4. These are assumed to have been at stages 41 and 43 respectively at the time of treatment, while stage 44 embryos would have been treated at stage 42. Therefore all of the stages treated are assumed to have been competent to respond to L-T_4. However, 72 hours after L-T_4 treatment, stage 44 embryos had phosphatase activities comparable to those of controls at stage 44. Therefore treatment before stage 42 appears to have resulted in a transient phosphatase increase, while treatment at 42 or 43 produced increases which fell within the normal time course.

These results are consistent with those of Moog and Richardson (1955), who found that precocious stimulation of alkaline phosphatase varied according

to the time of treatment with adrenocorticoids. Treatment before 16 days produced slight elevation; at 16 days it produced pronounced elevation within 48 hours; and at 17 or 18 days elevation occurred within 24 hours. They suggested that the embryo's period of competence to respond to corticoids is not much longer than the time in which intensive differentiation actually occurs. The same may be suggested as to the embryo's ability to respond to thyroxine treatment, since the magnitude and duration of response varied with stage.

When the results of the alkaline phosphatase determinations were pooled according to time (i.e. all stages encountered at one time with the same treatment were pooled), the phosphatase increase 48 hours after L-T_4 treatment remained significant. At 72 hours however, pooling the data in this fashion reduced the L-T_4 treated value to that of controls, due to the inclusion of the lower stage 44 values. Therefore, while pooling the data according to time emphasizes the fact that increases occurred in all stages at 48 hours, it de-emphasizes the effects at 72 hours. Thus staging of the embryos may present the phosphatase situation more accurately than when day of incubation alone is used.

The precocious increase in duodenal alkaline phosphatase seen here following thyroxine treatment is in contrast to Moog's results (1961b). She found that treatment of otherwise normal embryos with thyroxine on

days 13 and 15 (0.75 μg each day) failed to alter the normal pattern of phosphatase accumulation, while similar treatments could repair the intestinal effects of thiourea treatment. At 18 days, normal embryos treated with T_4 had higher alkaline phosphatase activities than controls, but this difference was not significant (ibid.), and Moog concluded that thyroxine must be permissive to the stimulatory effect of cortical hormone in the control of duodenal differentiation. In comparison with the present study, Moog's treatments may have been too early or the dosage too small to produce large increases in phosphatase. The first possibility seems supported by the period of competence found by Moog and Richardson (1955) with corticoid treatments. However, Mallon (1972) found increased phosphatase activity at day 16.5, following daily injections of 1.5 μg T_4 beginning on day 13.5, indicating that dosage may be a determining factor. (As his study was not carried on beyond 16.5 days, it is not known whether the increase was transient.) As well, Black and Moog (1978) found that explants of 14 day duodenum could respond to T_4 <u>in vitro</u>.

An additional possibility is suggested by comparison of the patterns produced by staged and time-pooled results, that Moog's (ibid.) thyroxine treatments did produce an increase in alkaline phosphatase in some stages, but the group was heterogeneous enough to mask this.

Effects of T_4 Treatment on Duodenal Protein Content

Twenty-four hours after L-T_4 treatment, total duodenal protein content of stage 43 embryos was elevated. This difference was significant with respect to all groups except the D-T_4 treated one. When the results were pooled according to time, the L-T_4 group remained significantly different from the windowed and saline-treated groups only. At later times, duodenal protein content did not appear to vary with L-T_4 treatment, although stage 45 L-T_4 treated embryos at 48 hours had lower values than stage 45 embryos at 72 hours. This suggests that incubation time and stage may both affect the increase in protein content. Therefore L-T_4 treatment appears to have produced an early increase in duodenal protein content, evident at 24 hours only.

The protein contents of the L-T_4 and D-T_4 treated stage 43 groups did not differ significantly, and the latter is believed to have little or no physiological activity in chicks (Reineke and Turner, 1945). In rats, D-T_4 has been shown to be about as effective as L-T_4 in stimulating amino acid incorporation into protein *in vitro* but not *in vivo* (Sokaloff and Kauffman, 1961). Similarly, in rats both isomers have been found to be equipotent stimulators of atrial protein synthesis *in vitro*, while L-T_4 is a much more potent stimulator of cardiac hypertrophy *in vivo* (Axelrod *et al.*, 1974). Both groups suggested that

the lesser physiological activity of D-T_4 could be due to more rapid clearance, as found in rats by Tapley *et al*. (1959). Possibly D-T_4 is not cleared rapidly in the chicken embryo, and here actually showed some effect. Interestingly, stage 42 embryos which had been treated 48 hours previously with D-T_4 had duodenal protein contents significantly greater than all other stage 42 groups except the L-T_4 treated one. This seems to support some activity for D-T_4 and of a broader nature than that observed with L-T_4 as the latter appears to have been effective only when administered at stage 42.

Fresh and dry duodenal weights and body weights (Appendices VI and VII) showed very similar patterns 24 hours after treatment to those exhibited by duodenal protein at stages 42 and 43. Moog (1961b) found body weights of normal embryos not to be significantly affected by T_4 treatment, while thiourea greatly retarded body weight gain but did not affect the increase in duodenal protein until after 19 days. As protein content and alkaline phosphatase activity appeared to increase independently in the present experiments, the increase in protein content with T_4 treatment might be related to gain in muscle mass, explaining the correlation with the weight data. This could also account for the possible activity of D-T_4, perhaps it is similar to its stimulation of cardiac hypertrophy (Axelrod *et al*., 1974).

If protein content was increasing more rapidly than alkaline phosphatase 24 hours after treatment, then the specific activities observed in the D-T_4 groups could have been proportionately lower. However, the similarity of phosphatase among all groups does not support this.

Factors Contributing to Structural Variation in the Duodenal Epithelium

Hilton (1902) first noted that individual variation in the progress of duodenal morphogenesis in chicken embryos was very great. In the mature intestinal epithelium cells are constantly renewed through mitosis in the crypts, migration along the villus, and extrusion at the tips (Leblond and Stevens, 1948). While extrusion of cells does not begin until after hatching, by day 16 mitoses have become localized at the bases of the villi, beginning the establishment of the mature cycle (Hinni and Watterson, 1963). The distribution of alkaline phosphatase also increases from villar base to tip (Moog, 1950; see Moog *et al.*, 1969). Therefore observations were limited to the upper thirds of villi, where cells of the greatest maturity would be found.

The tallest villi were of the most mature appearance, and these according to the observations of Grey (1972) were Set I villi. He suggested that most previous morphological studies in the chicken embryo have dealt with this rank although this could not be verified.

Therefore, the present observations are suggested to be comparable in villar location to those of previous studies. As yet a detailed study relating development of villus architecture to epithelial differentiation has not been reported.

Longitudinal variation in the duodenum may be contributing to variation. Ridge formation proceeds from proximal to distal (Hilton, 1902). In mouse and rat, alkaline phosphatase activity has been found to be greatest in the duodenum and to decrease proceeding distally through the jejunum and ileum (Moog, 1961a; Harrison and Webster, 1971; Laval et al., 1978). Hancox (1954) found that histochemically demonstrable alkaline phosphatase first appeared in the distal loop of the chicken embryo duodenum, however the possibility that gradients in phosphatase and cytodifferentiation exist has not been further investigated. Determinations using duodenal quarters in the present experiments (Appendix VIII) revealed no significant differences in alkaline phosphatase activity or total protein, however the possible establishment of a proximal to distal gradient after stage 45 was suggested by increased proximal phosphatase.

For morphological examination duodenal samples were all taken from the proximal quarter in the experiments performed in vivo, therefore should be equivalent. For the cultured material however different segments were used for structural examination in order to preserve the

random nature of the experimental design. Preliminary observations indicated that the different segments were morphologically comparable following 48 hours in culture, though Hancox (1954) had found that distal duodenal fragments did not progress as well as proximal ones following 48 hours in culture.

Effects of Thyroxine Treatment on Morphology of the Duodenal Epithelium

When chicken embryos were treated with corticoids, acceleration of alkaline phosphatase accumulation was accompanied by accelerated structural differentiation of the duodenal epithelium (Moog and Richardson, 1955). Similarly, adenohypophyseoprivia induced by partial decapitation slowed cellular development, and replacement therapy restored differentiation at both cellular and enzymatic levels (Hinni and Watterson, 1963; Bellware and Betz, 1970; Hart and Betz, 1972a,b). Examination of the morphology of duodenal epithelium from the experiments performed *in vivo* revealed an increasingly mature appearance accompanying L-T_4 treatment, however these changes appeared to be more specifically related to certain cellular features rather than indicating uniformly accelerated development of the entire absorptive cell.

The changes observed during normal differentiation of the epithelium agree with those reported by previous investigators (Overton and Shoup, 1964; Dobbins *et al.*, 1967; Penttilä and Gripenberg, 1969; Ono, 1973).

Golgi Activity

The Golgi apparatus has been shown to participate in glycocalyx and cell coat formation in intestinal absorptive cells (Bennett, 1970; Bennett and Leblond, 1970; Bennett *et al.*, 1974). Much evidence has accumulated which indicates that the Golgi apparatus participates in the biosynthesis of membrane components including glycoproteins, glycolipids, phospholipids, neutral lipids and membrane proteins, and participates in the modification and transportation of membrane components (see review by Morré, 1977).

Alkaline phosphatase is a glycoprotein (Saini and Done, 1970; Forstner, 1971), normally associated with the outer microvillar membrane and glycocalyx (Holt and Miller, 1962; Overton *et al.*, 1965; Hugon and Borgers, 1966, 1969a,b; Ito, 1969; Ono, 1973; Louvard *et al.*, 1975). It has also been histochemically located in the Golgi complex (Moog, 1950; Hugon and Borgers, 1969b), although sometimes this location has been negative (Hugon and Borgers, 1969a). From these studies therefore it is suggested that the Golgi complex may be involved in alkaline phosphatase synthesis.

Yeh and Moog (1975a,b) observed that reduced Golgi activity and thyroxine levels in hypophysectomized rats were restored by T_4 treatment, and suggested that these parameters are all related. Similarly in the present experiments, embryos showed increased Golgi

activity in their duodenal absorptive cells 48 and 72 hours after L-T_4 treatment, paralleling the precocious increase in alkaline phosphatase. In normal cells, Golgi activity also increased with time, although not to the extent seen in L-T_4 treated cells, therefore a normal increase in Golgi activity may be related to the normal increase in phosphatase.

Lysosomal Activity

The Golgi complex has also been shown to be involved in lysosome production in intestinal absorptive cells (Moe et al., 1965; Bennett and Leblond, 1971). The lysosomal system has been reviewed extensively (e.g. DeDuve and Wattiaux, 1966; Wattiaux, 1969; Dingle and Fell, 1969; Dingle, 1972; Holtzman, 1976). According to these authors, primary lysosomes (dense bodies) originate from the Golgi and smooth endoplasmic reticulum, with participation of the RER as well (Cohn et al., 1966); the primary lysosome is a storage granule until it meets a pre-lysosome or phagosome in the form of an endocytotic vesicle or multivesicular body and fuses with it (Cardell et al., 1967), becoming a secondary lysosome where digestion of the phagosomal material occurs. Thus the increase in secondary lysosomes observed with T_4 treatment is consistent with an increased digestive activity.

It now appears that the lysosomal system may function in the re-cycling of membrane materials: secretory

vesicles carry membrane components from the Golgi to the cell surface, and endocytotic vesicles carry membrane back into the cell and membrane components are recovered and the cycle repeated (see Morré, 1977). Consistent with this possible cycling activity is the histochemical localization of alkaline phosphatase in the apical pits and vesicles, smooth surfaced endoplasmic reticulum, multivesicular bodies and lysosomes of chick duodenal absorptive cells (Hugon and Borgers, 1969a).

Therefore, the increase in secondary lysomal activity seen following L-T_4 treatment is consistent with increased turnover of membrane components. Normally, the cell coat and brush border membrane are in a constant state of renewal (Bennett, 1970; Bennett and Leblond, 1970; Bennett *et al.*, 1974; Jersild and Crawford, 1978) and lysosomal activity may be related to the recycling of these components of the brush border.

Endocytosis

In the present experiments, apical pits and vesicles, and multivesicular bodies were seen at all stages and with all treatments. In intestinal absorptive cells these structures are all associated with endocytotic activity (e.g. Clark, 1959; Palay and Karlin, 1959; Cardell *et al.*, 1967; Porter, 1969). Moog and Yeh (1975a,b) found that hypophysectomy reduced endocytosis, and T_4 treatment restored it in rat duodenum. Here

endocytotic activity was not observed to increase with T_4 treatment, however the nature of these vesicles makes quantitation difficult without the use of markers. Palay and Karlin (1959) calculated that a few apical pits and vesicles seen in a section of a single cell would represent a great deal of activity when the entire apical surface was taken into consideration.

In fetal mammals, endocytosis is apparently involved in the absorption of antibodies from swallowed amniotic fluid by jejunal and ileal absorptive cells (Orlic and Lev, 1973). In suckling mammals large vacuoles form in the apical cytoplasm of these cells, apparently as a result of endocytosis (Clark, 1959; Cornell and Padykula, 1969; Walker et al., 1972; Moon et al., 1973; Ono and Takashio, 1978). Alkaline phosphatase has been localized on the periphery of these vesicles (Clark, 1961; Ono, 1975).

In chicken embryos a mixture of albumen and amniotic fluid is injested, mainly between 11 and 15 days of development (Witschi, 1949; Carinci and Manzoli - Guidotti, 1968). While dietary albumen may ordinarily stimulate duodenal development (Betz and Hart, 1977), evidence for actual absorption of albumen components by the duodenal epithelial cells is lacking. Kramer and Cho (1970) found increased antibody titres in intestinal homogenates during the last week of incubation, but whether this represents absorptive activity is uncertain.

If albumen absorption in chicken embryos is similar to the pattern seen in mammals then the jejunum and ileum may be more directly involved in its utilization.

Increased endocytosis has been observed in connection with several absorptive processes in intestinal epithelial cells: lipid absorption (Palay and Karlin, 1959), vitamin B_{12}-intrinsic factor absorption (Mackenzie and Donaldson, 1969), and calcium absorption (Jande and Brewer, 1974). In the case of lipid absorption a role for endocytosis has been ruled out (Strauss, 1966; Cardell *et al.*, 1967; Porter, 1969) and its possible function in the other systems is speculative, rather it may be associated with these processes secondarily through increased membrane flow (Morré, 1977). Therefore the presence of endocytotic vesicles in the epithelial cells of chicken embryo duodenum cannot be interpreted as an indication that some particular absorptive system is at work. Endocytosis may however be operating in membrane flow (Morré, 1977) which would serve to recycle surface components as the brush border differentiates and acquires new membrane components.

Brush Border

The changes observed here in the developing brush border agree with the observations of previous investigators (Overton and Shoup, 1964; Penttilä and Gripenberg, 1969; Ono, 1973). While microvillar height

increased with advancing stage, quite a wide range of height was found at all stages and any differences in height which might be due to treatment could not be detected. Moog and Yeh (1975a) also found considerable variation in the microvilli of rat duodenum and did not detect any changes in the microvilli with hypophysectomy. Therefore while microvillar development parallels alkaline phosphatase accumulation (Overton and Shoup, 1964), increased phosphatase activity is not due simply to an increase in microvillar surface area.

Glycogen

Transient accumulation of glycogen has previously been observed in chicken embryo duodenum by means of histochemical and biochemical methods. The amount of glycogen present peaks at day 17.5 (Bellware and Betz, 1970) or 18 (Moog and Richardson, 1955; Moog and Thomas, 1957; Hinni and Watterson, 1963), then rapidly disappears from the cytoplasm, being virtually absent by day 20. Using whole intestine, Baxter-Grillo (1969a) found that glycogen peaked at day 20.

In the present experiment glycogen was seen most frequently at stages 42 and 43 (days 16.5 and 17.5), and thereafter appeared as small deposits, suggesting that quantities were decreasing. Thus these observations agree with those of the previously mentioned authors.

At stages 42 and 43 glycogen often appeared as discrete articles of great electron density, while later it was mainly flocculent and only slightly electron dense. These areas of pale-staining glycogen had the appearance of 'empty spaces' in contrast to the surrounding electron dense cytoplasm, but on closer examination the flocculent nature of their contents was evident. Similar observations of variation in the appearance of glycogen, and the difficulty in distinguishing scattered particles from ribosomes have been made in other tissues (e.g. Fawcett, 1958; Revel et al., 1960; Revel, 1964), and were suggested to indicate loss during fixation or differences in physiological state. These two possibilities may be related as Bellware and Betz (1970) observed that amylase extraction of PAS positive material (presumably glycogen) proceeded more rapidly at 18.5 and 19.5 days of incubation than at earlier times. Moog and Thomas (1957) found that duodenal glycogen is the physiological type, extractable with cold TCA.

Revel (1964) indicated that Millonig's (1961) buffer may be a superior vehicle for OsO_4 in the preservation of glycogen. In the present study, electron dense particulate glycogen appeared quite obvious in material post-fixed with this buffer, but pale flocculent glycogen also appeared.

In previous electron microscopic studies of chick embryo duodenum, only Dobbins et al. (1967) observed

electron dense, particulate glycogen: more at day 16 than day 20, the only two days of their study. Holman (1969) observed 'globules of varying size, bigger and of a lower electron density than ribosomes' adjacent to lipid droplets, but showed no electron micrographs of duodenum. A similar relationship between glycogen and lipid droplets was observed in the present study.

Glycogen deposition and depletion in the duodenum is apparently under the influence of hormones. In embryos treated with adrenocorticoids, alkaline phosphatase, the stimulated levels surpass the untreated values (Moog and Richardson, 1955; Moog and Thomas, 1957). In adenohypophyseoprivic embryos duodena glycogen fails to be depleted (Hinni and Watterson, 1963). In the experiments performed in vivo, less glycogen was seen in the cytoplasm of stage 44 and 45 embryos following treatment with L-T_4, indicating that this hormone may stimulate glycogen ultilization during duodenal development. However, in light of the variability and difficulty in observing glycogen, histochemical and biochemical corroboration of these observations are necessary before the effects of T_4 on duodenal glycogen can be stated.

Lipid Droplets

The large rounded inclusions present in the cytoplasm of developing epithelial cells of the embryonic chicken duodenum have been identified as lipid droplets

(Dobbins et al., 1967; Holman, 1969). In morphological studies, several authors have observed perinulcear vacuoles in the cytoplasm of the epithelial cells which would appear to be lipid droplets (Hinni and Watterson, 1963; Bellware and Betz, 1970; Hart and Betz, 1972a,b), and others have not indicated if they were observed (Argesneau and May, 1938; Penttilä and Gripenberg, 1969). Therefore they may be more common in the duodenal epithelium of some strains than others, or the resemblence of the droplets to vacuoles at the light microscope level may have caused their neglect.

Dobbins et al. (1967) observed more lipid droplets at 16 than 20 days of incubation. Similarly perinuclear vacuolations have been observed to diminish from 18 days onward (Bellware and Betz, 1970; Hart and Betz, 1972a,b) in normal embryos, but not in those made adenohypophyseoprivic by partial decapitation. By two days post-hatching no lipid droplets are seen in the intestinal epithelium (Holman, 1969).

Although the droplets were not counted, they diminished with increased stage, and were seen less frequently following L-T_4 treatment at stages 44 and 45. Therefore their utilization may be accelerated by T_4 treatment, possibly to provide an energy source for the differentiative changes.

Holman (1969) observed diversity in the appearance of the contents of the lipid droplets, but did

not study their appearance with increasing age. In the present study, the granules' contents generally decreased in electron density and their limiting membranes disappeared. Holman (1969) observed a type of membrane surrounding the droplets but pointed out that it was not a unit membrane, which seems consistent with the present observations. (His electron micrographs are not of duodenum therefore comparison is difficult.) He noted that these droplets form in cells poor in rough endoplasmic reticulum (Maunsbach and Wirsén, 1966), possibly because such cells are unable to utilize excess circulating fatty acids. In embryos, the yolk gives rise to such an excess (Noble and Moore, 1965). Possibly the intestinal cells then store lipid at a time when they cannot use it, for subsequent utilization during the days prior to hatching when a large energy source might be required to fuel the rapid structural and enzymatic changes. That the stores of yolk and glycogen are not depleted in adenohyphyseoprivic embryos (Hinni and Watterson, 1963; Bellware and Betz, 1970; Hart and Betz, 1972a) suggests that utilization of these substances is intimately linked to pre-hatching differentiation.

Ribosomes and Edoplasmic Reticulum

In the cells of the duodenal epithelium, rough endoplasmic reticulum was seen more often and as longer cisternae with increasing stage in all groups. Concurrently, free ribosomes became less common and groups

of polyribosomes were seen more frequently. These findings are similar to those of previous investigators (Dobbins et al., 1967; Penttilä and Gripenberg, 1969) and parallel light microscopic observations of decreasing basophilia thought to indicate a shift in the ribosomal population from free to membrane-bound (Moog and Richardson, 1955; Kato, 1959; Moog, 1961b; Hinni and Watterson, 1963; Hart and Betz, 1972a). A similar change in the transformation of crypt to columnar absorptive cells is accompanied by a decrease in RNA content, and is thought to indicate a shift to the adult protein synthesis organelle (Webster and Harrison, 1969). This change parallels the alkaline phosphatase increase in embryonic cells, and is probably involved in the sysnthesis of its protein component, as well as in the synthesis of other membrane proteins (Seetharam et al., 1977) required for functional differentiation.

Agranular endoplasmic reticulum was also observed more frequently with increasing stage as in previous studies (Dobbins et al., 1967; Penttilä and Gripenberg, 1969). In intestinal absorptive cells it has been shown to be involved with lipid absorption (Cardell et al., 1967) therefore it seems possible that SER may be participating in the mobilization of the lipid droplets. Possibly the increased RER also participates in or enables the utilization of the lipid droplets, as lack of RER is suggested to lead to their

formation (Holman, 1969). In liver cells SER is involved in glycogen depletion (see Cardell, 1977), therefore that it may perform a similar function in the developing duodenal epithelial cells is suggested by the observation that the increase in SER coincides with the decrease in glycogen.

Granular endoplasmic reticulum is also involved in lysosome formation through the synthesis of acid hydrolases, and SER participates in their transport; the degree of participation of each of these elements seems to vary depending on the involvement of Golgi in lysosome formation in a particular cell type (see Holtzman, 1976; Morré, 1977). As well, the synthesis and renewal of Golgi membranes may occur through a similar system of synthesis on ribosome-studded endoplasmic reticulum and transport via smooth surfaced elements (Morré, 1977). Therefore it seems that all of these organelles may participate as a system in the functional differentiation of the epithelial cell.

The agranular endoplasmic reticulum also participates in peroxisome formation in intestinal epithelial cells (Novikoff and Novikoff, 1972). In the present study peroxisomes were possibly present, but not yet distinguishable from dense bodies, which they resemble quite closely. Possibly a histochemical study, such as that done by Calvert and Ménard (1978) in developing mouse intestine would provide further information.

In hypophysectomized rats RER was found to be sparse (Yeh and Moog, 1975b). Here changes in endoplasmic reticulum and ribosomes did not appear to be affected by L-T_4 treatment. Such comparisons were hindered by lipid droplets and glycogen, as cells without these components would appear to possess more endoplasmic reticulum due to its presence in areas which might otherwise be occupied by inclusions.

Experiments Performed *In Vitro*

Culture Technique

Duodenal explants from chicken embryos have previously been cultured successfully on grids (Hayes, 1965a,b; Corradino, 1973). Similar methods have also been used for the culture of adult intestine from several species including: human (Browning and Trier, 1969; Hauri *et al.*, 1975), rabbit (Kagnoff *et al.*, 1972), and guinea pig (Kedinger *et al.*, 1974). Browning and Trier (1969) suggested that culturing the intestine slit open on a grid provided better oxygenation of the villar surface by capillary flow than possible during total immersion in the culture medium. While there is some cellular migration around the cut edges, the tissue does not grow into or spread over the grid surface. Gravitational effects possibly contribute to shortening of the villi (Corradino, 1973), a characteristic which was observed here.

McCoy's 5A, the chemically defined culture medium used in the present study, has previously been used successfully for the culture of chicken embryo duodenum without the addition of serum or embryo extract (ibid.). Struthers (1970) found that calf serum accelerated alkaline phosphatase accumulation and histological development of duodenal explants cultured *in vitro*, therefore the use of such additives was avoided.

Bovine serum albumin was added to a set of cultures to provide an additional nutrient source, however these did not show any indications of stimulated development. This contrasts with the suggested stimulation of duodenal development by albumen ingestion *in vivo* (Betz and Hart, 1977), although whether these two situations could be considered equivalent is not known.

Morphology of Duodenal Explants Cultured *In Vitro*

The morphology of the absorptive epithelium of duodenal explants cultured *in vitro* for 48 hours was extremely variable. The cells ranged from normal in appearance to a form which was referred to as 'condensed' due to its increased cytoplasmic staining when examined by light or electron microscopy, nuclear condensation, and alterations in cell shape. The changes in cellular morphology may be due to culture conditions and/or changes due to fixation. Other causes of variability have been presented preceding discussion of ultra-

structural changes during the experiments performed *in vivo*, and the reader is requested to refer to that discussion.

The method of fixation used for the cultured material was developed by Buckley (1973) for cultured chick embryo cells. In preliminary experiments this method gave consistently better results than with the fixation used in the experiments performed *in vivo*, but probably more trials are warranted.

Buckley (1973) found that the mitochondria were easily damaged during fixation, and developed vacuoles and other signs of structural damage. In the present study, and in the observations of Dobbins *et al.* (1967), enlarged and unusually shaped mitochondria with bizarrely arranged cristae were seen, however there was little evidence of mitochondrial vacuolization.

Franks and Wilson (1977) in reviewing the ultrastructural changes exhibited by cells cultured *in vitro*, pointed out that mitochondrial changes of these types often appear, and suggested that they are due to changes in the cells' respiratory pathways. These authors also noted that conditions ideal for mitochondrial fixation usually have not been used in culture studies, and suggested that a mixture of glutaraldehyde and paraformaldehyde is a superior mitochondrial fixative compared to either glutaraldehyde or osmium alone for primary fixation. Dobbins *et al.*

(1967) used osmium, and the present study used glutaraldehyde as the primary fixative. Therefore it appears that the changes observed in mitochondrial structure cannot be attributed solely to either culture or fixation. Similarly, changes in other organelles may result from either or both sets of conditions.

Many cells, when observed following culture, showed ultrastructural changes which were suggestive of possible condensation of cytoplasmic components. Nuclei were sometimes full of condensed chromatin, and even compacted in size, suggesting that they had probably become transcriptionally inactive (see Wischnitzer, 1973; Franks et al., 1977). Ribosomes were often packed into groups which probably contributed to the strong basophilia shown by these cells when stained with toluidine blue and examined light microscopically (cf. Moog and Richardson, 1955; Kato, 1959; Moog, 1961b; Hinni and Watterson, 1963; Hart and Betz, 1972a). Endoplasmic reticulum, Golgi, and lysosomes were greatly reduced, indicating that normal activities were probably reduced. However lack of extensive lysosomal (autosomal) activity indicates that the cells were not yet in a state of destruction (Holtzman, 1976).

Cells showing condensation of their cytoplasmic components were often elongated and possessed many lateral interdigitations, characteristics reminiscent of uncultured cells as they become increasingly columnar. These cells were often normal in the form of their apical regions.

These changes suggest that normal changes in cellular shape occurred in culture, but that possibly the rest of the cell's activities could not 'keep up' with these differentiative changes. Possibly metabolic changes as evidenced by altered mitochondria had occurred. Thus cytoplasmic components and organelles would not be synthesized or renewed quickly enough for cytoplasmic differentiation to parallel development of form. Perhaps supporting this speculation is the observation that absorptive cells which resembled the normal situation following culture were generally low columnar and so had not changed greatly in form.

Villi were often observed to be shortened, possibly due to the absence of cortisone which promotes increased villar height (Hayes, 1965a) as well as due to the presence of gravitational effects. Spooner et al. (1977) found that cytodifferentiation and morphogenesis can proceed independently in the pancreas. Therefore changes in villus architecture would not necessarily interfere with the differentiation of the epithelium.

Brush Border of Cultured Epithelium

In all cultures the absorptive cells exhibited intact brush borders, formed of microvilli of normal appearance. Development of the brush border often appeared to have progressed, although microvillar height had not increased. In cells which were very electron

dense, the core filaments could not always be discerned, but form was normal. In some areas microvilli appeared to be scarce, possibly because they were very short and not many would appear in a single section. However it seems that in these regions microvilli may have actually been 'lost' from the surface. As disruptions and blebbing of the surface to form vesicles were seldom observed, it would appear that in these areas microvillar shortening had occurred through failure of sufficient membrane synthesis to retain their form.

Hayes (1965b) observed a thick layer of vesicles, apparently derived from the microvilli, in 19 day chicken embryo duodenum cultured with cortisone. Elongated and branched microvilli, showing a similar vesicular appearance, were found in cultured fetal rat jejunum following 72 hours in culture (DeRitis et al., 1975), and in chicken embryo duodenum cultured in the presence of cytochalasin B, a disruptor of microfilament organization (Burgess and Grey, 1974). Similar (temperature-dependent) vesiculation in isolated kidney brush borders has been attributed to breakdown of cross-bridges between the membrane and actin filaments (Booth and Kenny, 1976). Black and Moog (1978) have indicated that microvilli were elongated and that alkaline phosphatase rich vesicles were released into the culture medium. The details of these are as yet unpublished. Therefore it seems that the brush border can become disrupted for various reasons in culture, however the

significance of this is not known.

The only consistent change in the apical surface of the present cultures was the presence of a thicker glycocalyx in cultures with 14 ng L-T_4/ml of medium, in cultures with and without BSA. Ito (1969), using the very thick glycocalyx of cat intestinal epithelium found that alkaline phosphatase was histochemically located only in the portion of the glycocalyx immediately adjacent to the membrane. Therefore changes in thickness of the glycocalyx would probably not be quantitatively related to phosphatase. It could however indicate the stimulation of another component of the glycocalyx not associated with the membrane, e.g. disaccharidases (Johnson, 1969). Black and Moog (1978) found that T_4-stimulated increased maltase in cultured chicken embryo duodenum.

In all cultures a glycocalyx was present on the apical surface, although it was sometimes reduced to a thin fuzzy coating. Therefore it is suggested that the normal site of alkaline phosphatase remained intact.

Goblet Cells

In contrast to the absorptive cells, goblet cell differentiation seemed to proceed at a faster rate in cultured explants than normally occurred *in vivo*. Following 48 hours in culture, mature goblet cells possessing a prominent mucous-filled theca and well

differentiated foot region were plentiful. In the comparable stage of normal development (44) goblet cells were in an intermediate state (Freeman, 1966), characterized by few mucous droplets and an overall cytoplasmic appearance and shape quite similar to that of the absorptive cells.

Black and Moog (1977) found that goblet cell numbers increased more rapidly than in vivo, in explants of 14 day chicken embryo duodenum cultured 48 hours. Thyroxine accelerated this increase while hydrocortisone prevented it (ibid.). Similarly goblet cell differentiation and migration were found to be inhibited in partially decapitated embryos (Hinni and Watterson, 1963; Bellware and Betz, 1970) and these defects could be restored by grafts of pars distalis (Hart and Betz, 1972a). Black and Moog (1977) suggested that an inhibitor of goblet cell differentiation possibly exists in vitro, and that removal of the duodenum from its presence by explanting would then have a stimulatory effect. Cortisone was suggested as a possible inhibitor since it inhibited the increase in goblet cells in vitro.

The accelerated differentiation shown by goblet cells here could be interpreted as supporting the inhibitor theory, especially since goblet cells were well differentiated in all cultures. However they were not counted, therefore whether T_4 affected their numbers cannot be stated. Superficially however, goblet cells appeared to be equally plentiful in all cultures.

This suggests that increased prominence of goblet cells may be more directly related to the presence of culture conditions which are favourable to them. Initially goblet cells and absorptive cells resemble each other ultrastructurally (Freeman, 1966). Whether the differentiative pathway which a cell is to follow can be changed as it migrates up the villus, or whether its fate is fixed as it is produced, is not known.

The resemblance of condensed absorptive cells, produced during culture to the differentiated foot of the mature goblet cell, suggests that culture conditions which seem to shut down normal differentiation in the epithelial cells have a similar effect on the goblet cells. However in the latter case these conditions are actually mimicing normal differentiation and thus accelerate their development. Culture conditions are not always favourable to goblet cells however, as they have been observed to decrease in number and show poor morphology in explanted guinea pig and mouse duodenum (adult) cultured with Trowell medium (Kedinger et al., 1974; Ferland and Hugon, 1975).

Biochemical Results

Following 48 hours of culture in the presence of 14 ng L-T_4 per 100 ml of medium (no BSA) alkaline phosphatase specific activity of duodenal quarters was significantly elevated. In cultures containing BSA

this concentration was not effective in elevating phosphatase, while 14 ng L-T_4/ml produced a smaller increase.

The concentration 14 ng/100 ml is equivalent to the free circulating T_4 level found in 17 day chicken embryos by Davison (1976). It has been suggested that the free circulating T_4 level is the true index of thyroid activity in several vertebrate species (including chickens) while bound T_4 is not a good indicator (Refetoff et al., 1974). As thyroid hormones normally bind to chicken serum albumin (Tritsch and Tritsch, 1965), it could be suggested that a higher L-T_4 concentration was required to stimulate alkaline phosphatase in the presence of BSA due to binding of hormone by BSA.

Black and Moog (1978) found a range of L-T_4 concentrations to be effective in stimulating alkaline phosphatase accumulation in explanted duodena: 10^{-10} M was effective in (7.7 ng/100 ml) the stimulation of 12, 14, and 16 day but not 18 day duodena; the maximum response was found with 10^{-8} M, and the effects declined at 10^{-7} M. Struthers (1970) previously found T_4 to retard morphogenesis and alkaline phosphatase accumulation in organ cultures of duodenum, however he used a much higher hormone concentration (0.5 μg/ml).

The results of the present study, that the presence of L-T_4 in the culture medium can be accompanied by increased alkaline phosphatase specific activity in

explants of embryonic duodena, agree with the results of Black and Moog (1978). They found that hydrocortisone alone elevated phosphatase activity (14 days at explant) during the first 48 hours of culture, but thereafter T_4 was required to continue the increase (ibid.). Previously cortisone had been found to stimulate phosphatase precociously in 16 day explants (Moog and Nehari, 1954), but this effect was decreased when the duodena were older than 18 days (Moog and Kirsch, 1955). Similarly Hayes (1965b) found that response to corticoids was maximal in 15 and 17 day explants cultured for 48 hours. Therefore it seems that both hormones can affect the duodenum directly and apparently both can stimulate duodena of the same age. This agrees with the results of the experiments *in vivo*, where response to L-T_4 treatment occurred within the same period of competence which Moog and Richardson (1955) had found for response to corticoids.

Black and Moog (1978) found that withdrawl of T_4 from the medium after 48 hours slowed the acceleration in phosphatase accumulation to the normal rate of increase. This tends to support the findings of the experiments *in vivo* which indicated that treatment at stage 41 was followed by elevation of phosphatase before it decreased to the normal levels. As the circulating levels of thyroid hormones normally increase during this time (Davison, 1976; King *et al.*, 1977; Thommes and Hylka, 1977; Thommes *et al.*, 1977), a sustained level of thyroid

hormone may be necessary to maintain the alkaline phosphatase increase. Since free and total T_4 peak at day 16.5 to 17 (Davison, 1976; Thommes et al., 1977) while T_3 rises continuously to hatching (King et al., 1977; Thommes and Hylka, 1977), the former could provide a cue for the timing of the increase, and the latter could support or maintain the increase. Thommes and Hylka (1977) found total plasma T_4 to peak at 19.5 days, which does not support the possibility that T_4 provides an initiating cue, but does support a maintenance role. Among these studies only Davison (1976) measured free circulating hormone levels, therefore these comparisons may not be equivalent.

Effects of D-T_4 on Duodenal Explants

Duodenal explants cultured with D-T_4, with or without BSA exhibited high protein contents and low alkaline phosphatase specific activities. These findings did not vary depending on hormone concentration. The possibility that D-T_4 stimulated an increase in muscle mass is suggested by previous reports of its effectiveness in stimulating protein synthesis in vitro (Sokaloff and Kauffman, 1961; Axelrod et al., 1974), and by the finding of increased dry duodenal weight in explants cultured with D-T_4 (without BSA). An increase in muscle protein could lower alkaline phosphatase specific activities artificially, accounting for the low activities seen with

D-T_4. However, in previous studies D-T_4 and L-T_4 were equally effective *in vitro* (Sokaloff and Kauffman, 1961; Axelrod *et al.*, 1974), therefore it seems unlikely that they would exhibit different effects here.

Effects of Culture Medium on Biochemical Results

In the duodenal cultures, neither alkaline phosphatase specific activity or protein content of the explants increased as in the normal uncultured situation. In general, following 48 hours of culture, protein contents were slightly depressed and alkaline phosphatase values were similar to those determined at the time of explant. As well, fresh and dry weights had decreased following culture. Similar trends for all these values were found in preliminary experiments involving the culture of stage 42 and 43 explants for 24 and 72 hours. Therefore with respect to these criteria the explants did not appear to be differentiating, however the uniform resemblence to stage 41 to 42 indicates that the cultures were in a state of maintenance, in which they could respond to hormonal stimulation, but not to the extent seen *in vivo*.

This is in contrast to the recent findings of Black and Moog (1978) who found that duodena explanted from 14 day chicken embryos accumulated alkaline phosphatase at a faster than normal rate for 48 hours, but failed to continue to increase at an accelerated rate unless T_4 was added to the medium (199). They suggested

that an inhibitor of phosphatase increase may normally be present in the duodenum and that it is removed by explanting. Insulin, which is present in the duodenal epithelium (Baxter-Grillo et al., 1973) was suggested as a possible candidate for this inhibitor. In contrast, alkaline phosphatase increased in cultures of adult human jejunum in the presence of insulin, apparently without being affected by its concentration or breakdown (Hauri et al., 1975).

Previously Moog and Kirsch (1955) had found that alkaline phosphatase accumulation proceeded faster in duodenal explants cultured with medium 199 than in vivo. Hayes (1965a,b) found that cortisone treatment was necessary to maintain normal differentiation in duodenal explants, including increased alkaline phosphatase cultured with medium 199 and calf serum. Struthers (1970) also used 199 but did not indicate that it stimulated development, however he cultured for 4 to 6 days before examination so that stimulation comparable to that found by Black and Moog (1978) could have occurred earlier.

Medium 199 and McCoy's 5A differ in composition, the former being more 'complete'. Among the substances contained in the former, but not the latter, is vitamin D_2. Chicken embryo duodenum cultured in vitro can respond to the addition of the normal metabolite acting at the intestinal level, 1,25-$(OH)_2D_3$, as shown by increases in calcium absorption and alkaline phosphatase (Corradino,

1973; Yoshizawa and Moriuchi, 1976; Yoshizawa et al., 1976). As well, increased phosphatase is found in response to vitamin D_3, possibly due to conversion to the active metabolite within the duodenum (Corradino, 1973; Yoshizawa and Moriuchi, 1976). While D_3 is a more effective anti-rachitic agent than D_2 in chicks (Steenbock et al., 1932; Chen and Bosmann, 1964), the possibility that vitamin D_2 could also stimulate increased alkaline phosphatase in embryonic duodena cultured in vitro should be considered. Its concentration in medium 199 (0.1 mg/l, approximately 252 nM) approaches the concentration of vitamin D_3 (325 nM) shown to increase alkaline phosphatase significantly in explants of 20 day duodenum cultured in McCoy's 5A (Corradino, 1973). Duodenal explants as young as 15 days will respond to D_3, although this has not been studied in detail (ibid.). While vitamin D_3 is not generally believed to stimulate duodenal development, Yoshizawa et al. (1976) found that cortisone did not stimulate precocious phosphatase increase in explanted duodena from 14 to 20 day embryos, while a synthetic active form of vitamin D_3 did stimulate phosphatase in 20 day explants. They suggested that cortisone indirectly influences the development of alkaline phosphatase while vitamin D_3 metabolites might regulate phosphatase induction more directly. Therefore further investigation of this problem seems to be necessary to clarify the conflicting results.

It would appear from the present experiments that McCoy's 5A allowed maintenance and differentiation to occur, but was perhaps nutritionally inadequate to provide 'normal' development and differentiation. This is supported by the observation of few lipid droplets and glycogen deposits in the condensed cells. Corradino (1975) has reported that for large scale organ culture of chicken embryo duodenum a better medium has been Waymouth 752/1 with additions of BSA and oleate.

Conclusions

The results of these studies support the theory that T_4 normally participates in the control of duodenal differentiation in chicken embryos, since: 1) it can accelerate the normal increase in duodenal alkaline phosphatase specific activity, and ultrastructural changes which may be related to this activity appear concurrently, and 2) it can stimulate increases in alkaline phosphatase in duodenal explants of similar stage to those treated *in vivo* in a chemically defined medium. While the exact role of T_4 in this process is still unknown, it appears to interact with corticoids and to act directly on the intestinal epithelial cells.

Receptors for thyroid hormones have been found in the nuclei, cytosol, and mitochondria of several target tissues (see reviews by Tata, 1975; Oppenheimer *et al.*, 1976), but as yet receptors for thyroid hormones

have not been reported in developing intestine. Intestinal cytoplasmic receptors for glucocorticoids have, however, been found in rats, and occur at significantly higher concentrations during periods of responsiveness to corticoids, i.e. the first two postnatal weeks (Henning et al., 1975). A temporal relationship has also been found between alkaline phosphatase development and the level of cytoplasmic and nuclear glucocorticoid receptors in fetal rabbit small intestine (Lee et al., 1976; Lee and Solomon, 1978). In anterior pituitary, corticosterone and thyroxine have been reported to have inverse effects on glucocorticoid binding sites (Koch et al., 1978).

Black and Moog (1978) have suggested that thyroid hormones are necessary to complete pre-hatching differentiation. The present findings suggest that their role may be one of timing and/or initiating the completion processes in chicken embryo duodenum. Possibly elucidation of their intestinal receptors will reveal an interaction of corticoids and thyroid hormones at that level, which could explain their apparent ability to act at the same time. No doubt further information will be provided by study of the developing absorptive processes. It is still uncertain when duodenal absorptive and transport systems actually begin to function, whether they change in function as they develop, or what processes are actually developing. In light of these complexities, it is not surprising that the hormonal activities involved in controlling the development of this system are equally complex.

SUMMARY

The participation of thyroxine (T_4) in duodenal development in chicken embryos was investigated through a) experiments *in vivo* in which embryos were treated with T_4 following 16.5 days of incubation; and b) organ culture of duodenum from stage 42 embryos in the presence of chemically defined media (McCoy's 5A) containing different concentrations of D-or L-T_4, and in some cases BSA. The following results were found in the experiments performed *in vivo*:

1. The normal pre-hatching increase in duodenal alkaline phosphatase specific activity began 24 hours earlier in L-T_4 treated embryos than controls (normal intact, windowed, saline-treated, D-T_4 treated).
2. When the increase in alkaline phosphatase was stimulated more than 24 hours early it was followed by a decline.
3. Twenty-four hours after treatment, total duodenal protein was significantly elevated in L-T_4 treated and slightly elevated in D-T_4 treated embryos compared to the other groups.
4. L-T_4 treatment did not affect embryonic development in general as shown by the distribution of developmental stages, but did produce earlier yolk sac retraction and possibly earlier pipping of the shell.

5. The absorptive epithelial cells of L-T_4 treated embryos showed increased Golgi and lysosomal activity 48 to 72 hours following treatment, and earlier loss of lipid droplets and glycogen from the cytoplasm.

The following results were found in the experiments performed in vitro:

1. In cultures without BSA, alkaline phosphatase specific activity of duodenal explants was elevated significantly by the presence of 14 ng L-T_4/100 ml of medium, but not by 14 ng/ml compared to cultures containing D-T_4 or saline.

2. In cultures with BSA, alkaline phosphatase was elevated in cultures containing 14 ng L-T_4/ml, significantly compared to cultures containing 14 ng L-T_4/100 ml or D-T_4.

3. In both sets of cultures, explants cultured with D-T_4 had elevated protein contents and depressed alkaline phosphatase specific activities.

4. The ultrastructure of absorptive epithelial cells following 48 hours of culture varied from normal (similar to stage 42 to 44 uncultured cells) to 'condensed' in appearance.

5. Condensed cells exhibited increased cytoplasmic electron density, reduction in cytoplasmic area, elongated form, and numerous lateral interdigitations. They exhibited clumping of ribosomes, nuclear condensation, enlarged and abnormal mitochondria, and reduction in most organelles. Their brush border and terminal web regions were usually quite normal. These changes could not be

correlated with specific treatments.

6. A thick glycocalyx was found on absorptive cells cultured with 14 ng L-T_4/ml of medium (with or without BSA).

7. Goblet cell differentiation proceeded more quickly in organ culture than was characteristic _in vivo_.

From the results of these two sets of experiments, and the previous literature, it is concluded that thryoid hormones may normally participate in the control of duodenal development in chicken embryos, perhaps through direct action on the intestinal epithelial cells.

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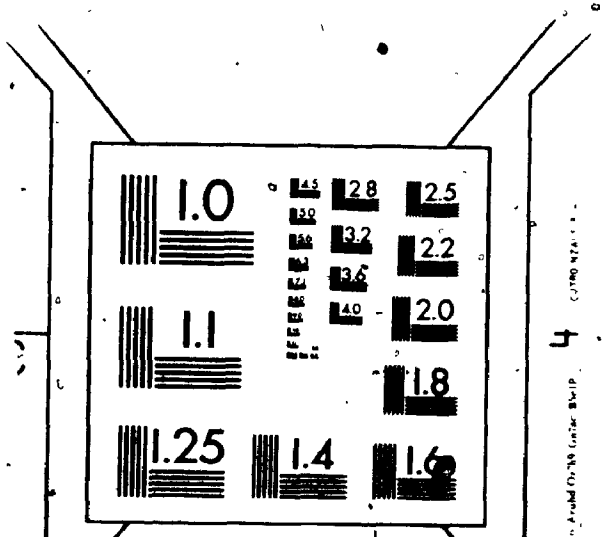

ARC - 374

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APPENDIX I

MATERIALS AND METHODS: SAMPLE CALCULATION OF TOTAL DUODENAL PROTEIN

The following formula was used for calculation of total duodenal protein as determined by the Lowry (1951) procedure (Materials and Methods III, 1 & 2):

Total Protein (mg) = OD x p x df ,

where OD = Optical denisity (560 nm),

p = conversion factor derived from slope of standard curve at 100 µg,

df = dilution factor,

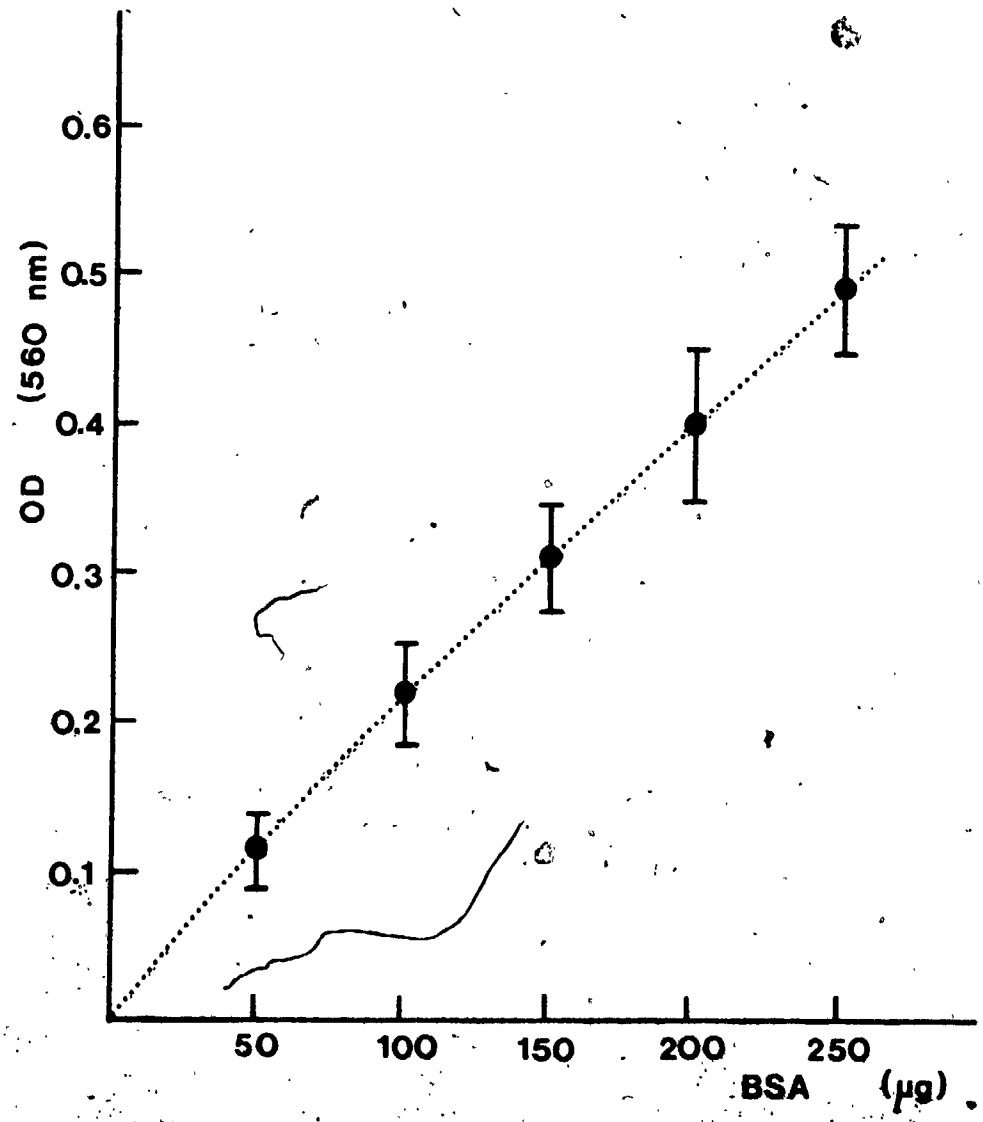
e.g. 0.5 ml of a 2.0 ml duodenal homogenate was used to obtain a protein sample which was dissolved in 10.0 ml NaOH. Two 1.0 ml samples of the 10.0 ml were used in the Lowry determination, giving optical densities of 0.15 and 0.16. Using the standard curve, 100 µg protein (BSA) was found to be equivalent to an OD of 0.20, thus p = 100/0.2 x 1 µg/1000 mg = 0.500. (The multiplication step is necessary to convert all units to mg.) Therefore the following calculation would be made:

Total protein = 0.155 x 0.500 x 40

= 3.1 mg.

The dilution factor of 40 is derived from the use of 1 ml of 10 to obtain an OD, the 10 ml containing one quarter of the original homogenate's protein i.e. 10 x 4 = 40.

Fig. A-1. Bovine serum albumin standard curve for protein determination using the Lowry (1951) method. Each point represents the mean of 50 determinations ± the standard deviation. The curve is nearly linear within the range used (OD 0.1 to 0.3), therefore a single conversion factor was derived from the slope at 100 μg.


0.6
0.5
0.4
0.3
0.2
0.1
OD (560 nm)
50
100
150
200
250
BSA (µg)

APPENDIX II

MATERIALS AND METHODS: SAMPLE CALCULATION OF ALKALINE PHOSPHATASE SPECIFIC ACTIVITY

The following formula was used for calculation of alkaline phosphatase specific activity as determined by the Manning *et al.* (1967) modification of the Babson *et al.* (1966) technique, using phonelphthalein monophosphate substrate. (Materials and Methods III, 3):

$$\text{Alkaline Phosphatase Sp. Act. } (\mu g/hr/mg \text{ prot.}) = \frac{OD \times s \times df}{\text{Total Protein}} \times \frac{60}{t},$$

where OD = Optical density (550 nm),

s = conversion factor derived from slope of standard curve at 60 µg phenolphthalein,

df = dilution factor,

t = incubation time (min),

Total Protein = total duodenal protein of sample (determined using Lowry, M & M III, 1 & 2).

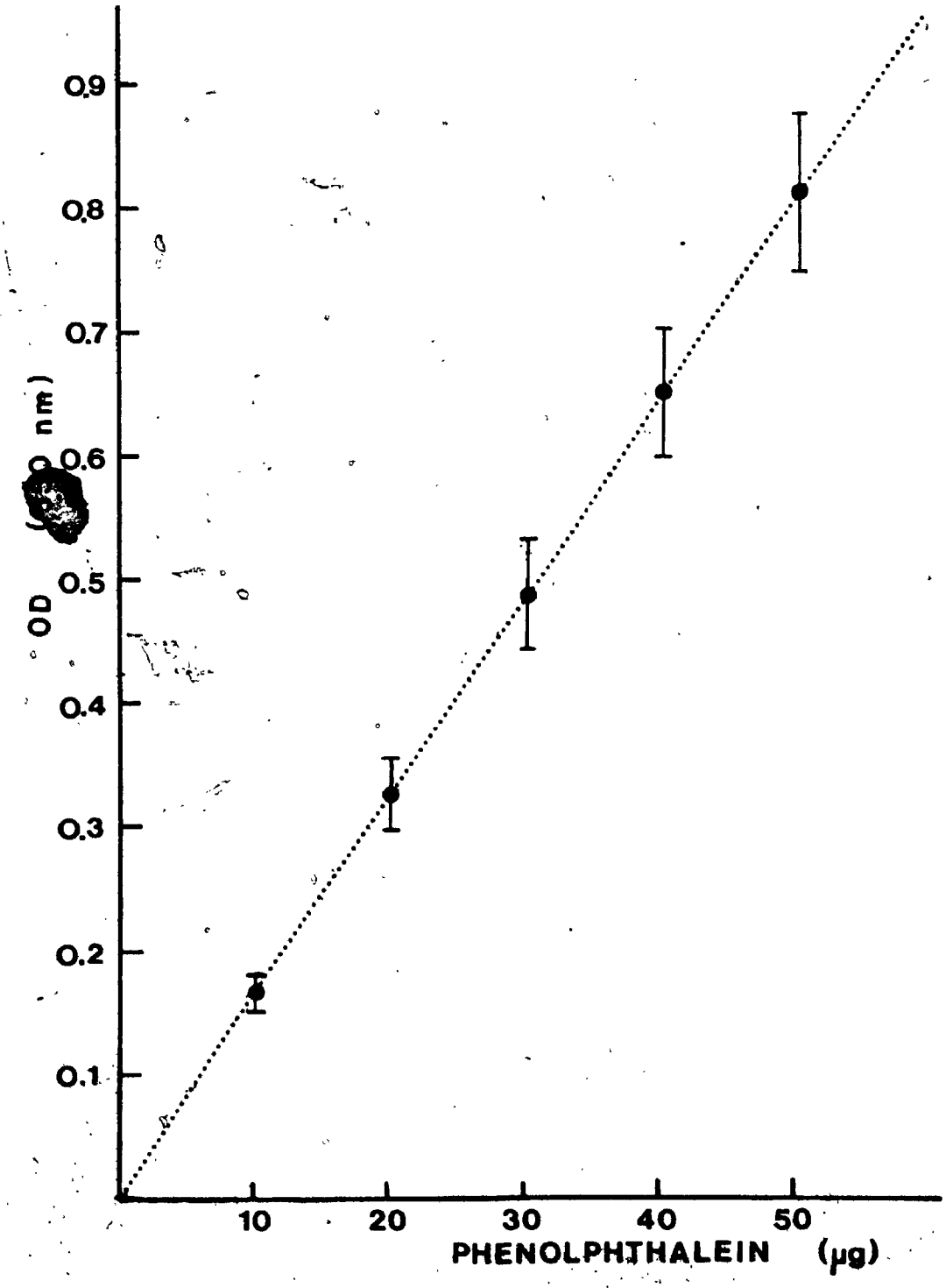
e.g. From the original 2.0 ml homogenate (Appendix I), 0.5 ml was separated for alkaline phosphatase determination. This sample was brought to a volume of 2 ml, of which 2 aliquots of 0.1 ml each were used for enzyme determination (df 4 x 20) yielding ODs of 0.15 and 0.16 @ 550 nm after an incubation of 20 min. Using the standard curve, 60 µg phenolphthalein was found to be equivalent

to an OD of 1.0 thus s = 60/1.0 = 60.0. Therefore alkaline phosphatase sp. act.

$$= \frac{0.155 \times 60.0 \times 80}{3.1} = \frac{60}{20}$$

$$= 720 \ \mu g/hr/mg \text{ protein.}$$

Fig. A-2. Phenolphthalein standard curve for alkaline phosphatase determination using the Manning *et al*. (1967) modification of the Babson *et al*. (1966) technique. Each point represents the mean of 80 determinations ± the standard deviation. The curve is approximately linear within this range. The slope at 60 μg phenolphthalein was used to derive a conversion factor for calculation of specific activity.


0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
OD
10
20
30
40
50
PHENOLPHTHALEIN (μg)

APPENDIX III

MATERIALS AND METHODS: ALKALINE PHOSPHATASE DETERMINATION USING ENZYME COMMERCIALLY PREPARED FROM *E. COLI*

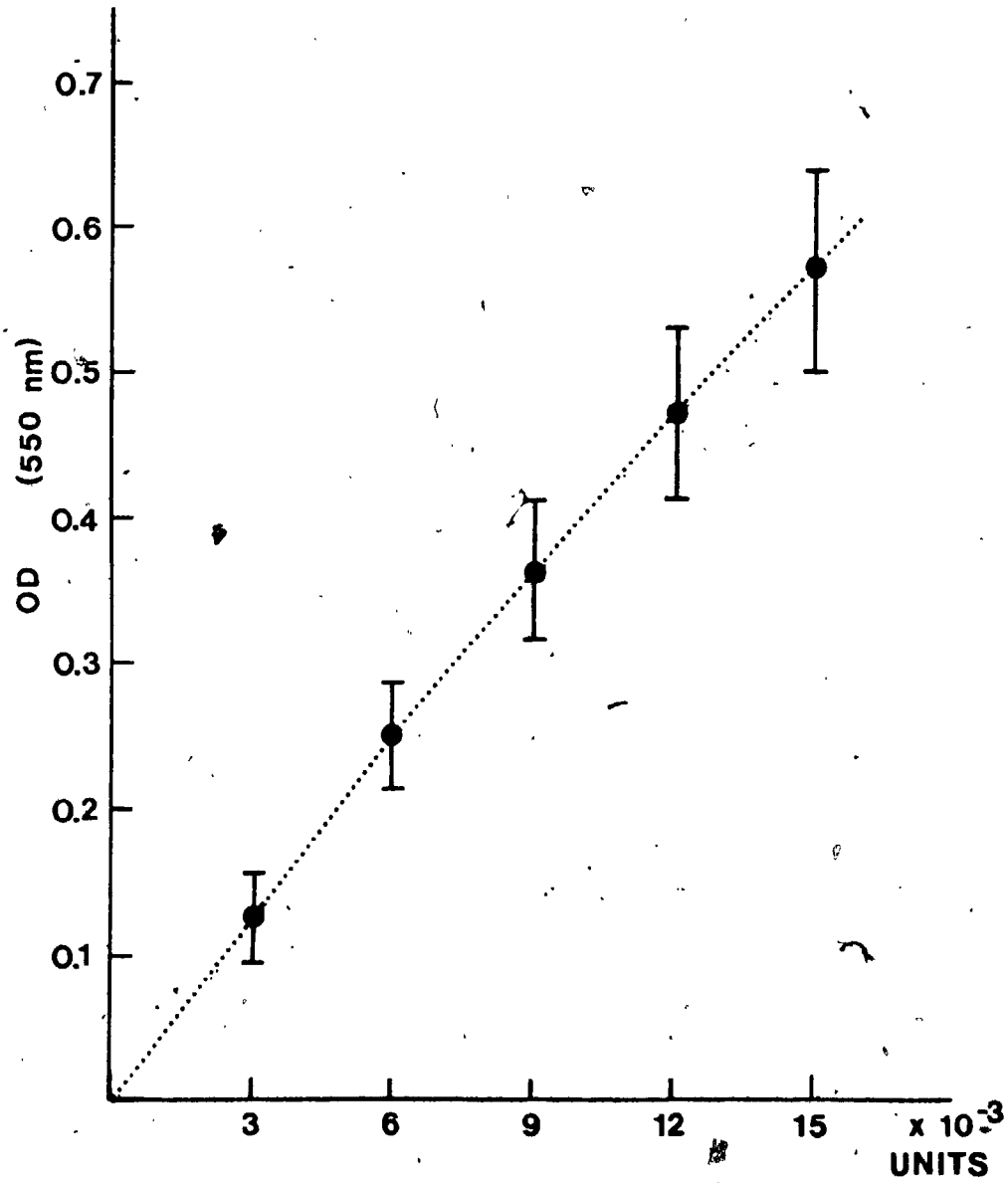
E. coli alkaline phosphatase (E.C. 3.1.3.1, type III-R, Sigma) defined as possessing 26 units of enzyme activity/mg protein and 5.8 mg/ml, was used to prepare samples containing 0.03 to 0.15 U/ml. Aliquots of 0.1 ml each were analysed using standard procedure (10 min incubation @ 37 $\pm$ 0.5^{o}C), producing a range from 3.0 to 15.0 x 10^{-3} U x 10 min.

One unit of enzyme will hydrolyze 1.0 µMole of free phenolphthalein or 318 µg. Calculation of expected phenolphthalein release during 10 minutes gave values of: 9.5, 19.08, 28.6, 38.2, and 47.7 µg. The obtained equivalents were: 7.5, 13.2, 21.6, 28.5, and 35.7 µg i.e. 72.3 to 84.9% of the expected values.

The protein content of the *E. coli* sample was defined as 5.8 mg/ml (Biuret) and found to be 5.6 mg/ml using the standard Lowry procedure.

Comparison of the curves produced with different runs of regular samples provided an indication that standard conditions were maintained.

Fig. A-III. Curve obtained using E. coli alkaline phosphatase (E.C. 3.1.3.1, type III-R, Sigma) in Manning et al. (1967) modification of Babson et al. (1966) technique for determination of alkaline phosphatase specific activity. Samples containing 3.0 to 15.0 x 10^{-3}U were incubated 10 min @ 37.5 $\pm$ 0.5°C. Each point represents the mean of 20 determinations $\pm$ standard deviation. Phenolphthalein release from phenolphthalein monophosphate approximately linear in proportion to enzyme concentration (Manning et al., 1967).


0.7
0.6
0.5
0.4
0.3
0.2
0.1
OD (550 nm)
3
6
9
12
15
x 10-3
UNITS

APPENDIX IV

RESULTS: DETERMINATION OF VARIATION DUE TO BIOCHEMICAL METHODS

Values for intestinal alkaline phosphatase specific activity in chicken embryos have been demonstrated to be variable (Moog and Richardson, 1955; Bellware and Betz, 1970; Hart, 1971; Hart and Betz, 1972b). Trial analyses were therefore performed in order to determine the amount of variation in results which could be attributable to the methods used rather than to variation among individuals.

Analysis I determined the variability introduced in the protein isolation procedure and in dilution prior to phosphatase assay. As summarized in Table A-1, a 2.0 ml homogenate was prepared from 10 duodena and then divided for subsequent analyses. Five aliquots of the homogenate were subjected to the protein separation procedures then dissolved in separate 10.0 ml volumes of NaOH. Each of these then provided four samples for Lowry protein determination. The mean of the calculated total protein values was 19.04 $\pm$ 0.3 mg (value A; see Table A-2). The standard error expressed as a % of the mean was 1.5%, which taken as an indication of the amount of variation introduced in the steps of the protein

purification procedure and subsequent assay, was much less than the actual variation encountered in the experimental data.

Three series of alkaline phosphatase determinations were performed using aliquots of the original 2.0 ml homogenate (Tables A-1 and A-2). In the first (Results B and C), five samples (0.1 ml each) were diluted in separate tubes and stored until the time of assay to provide an indication of variability due to the dilution procedure. A similar dilution factor was achieved by placing 0.5 ml homogenate in a single tube and bringing the volume to 5.0 ml; ten assays for alkaline phosphatase specific activity then provided an indication of variation due to the assay procedure (Results G and H).

As specific activity is calculated per mg total duodenal protein, variation could be introduced via the determination of protein content. Specific activities were therefore calculated in two ways: using the mean protein value (A) and individual phosphatase readings to derive Results B and G, and using individual protein values (of which A was mean) and the mean phosphatase readings to derive values C and H . In the first case variation would be due to differences in phosphatase OD, protein remaining constant, and in the second case variation would be due to differences in calculated protein, phosphatase remaining constant. The values

obtained for these calculations are shown in Table A-2. Expressing the standard error as a % of the mean, the greatest variation shown in these samples (B, C, G, H) was in G: 4.4% (variation due to assay, aliquots taken from dilution to one volume, protein remaining constant). None of these groups was significantly different from any other using the Student t test ($p>0.9$). Therefore variations in total protein did not greatly alter specific activity. The use of one dilution to provide many assay samples resulted in greater variation than did the use of several dilution tubes to provide two samples each. Possibly a result of dilution into a larger volume was less uniformity in addition to assay variability.

An additional assay was done to determine in what portion of the homogenate phosphatase activity was found. A 0.1 ml portion of the original homogenate (Table A-1) was brought to 1.0 ml then centrifuged at 700 x g for 10 minutes. Aliquots of 0.1 ml each were carefully removed from the supernatent and pellet fractions, avoiding re-suspension of the pellet. The supernatent was found to have significantly less alkaline phosphatase specific activity ($p<0.01$) than the pellet: 617.3 $\pm$ 12.1 μg/mg/hr, compared to 1104.5 $\pm$ 47.2 (Table A-2). The mean of the two fractions was 858.5 $\pm$ 93.5, not significantly different from the values obtained for

B, C, G, and H ($p > 0.9$). The supernatent value differed significantly from B, C, G, and H ($p < 0.05$), while the pellet did not differ significantly from these groups ($p < 0.05$). Therefore much of the homogenate's alkaline phosphatase specific activity was associated with the particulate portion which could be separated by centrifugation.

An additional trial, Analysis II, was done using a small sample of low specific activity from culture material. A duodenal sample was homogenized and the volume brought to 2.0 ml (Table A-3). A protein sample obtained from 0.5 ml of the homogenate by the usual procedure and dissolved in 10.0 ml NaOH was used for 10 Lowry determinations (1.0 ml each). The mean of these determinations was 2.3 mg $\pm$ 0.01. Ten separate alkaline phosphatase determinations were made using 0.1 ml aliquots of the original homogenate. The mean specific activity obtained (using 2.3 mg protein) was 44.8 $\pm$ 2.0 µg/hr/mg. In both instances the variation was small; expressing the standard error as a % of the mean, the variation in protein assay was 0.4% and in phosphatase assay 3.4%. These variabilities may be attributed to the assay procedures as dilution and other variables were kept to a minimum.

Table A-1 Summary of Sample Separation and Dilutions For Determination of Variation Due to Biochemical Methods I

10 lyophilized duodena pooled and homogenized in 1.0 ml distilled water; volume brought to 2.0 ml.

0.1 ml homogenate placed in each of 5 tubes and treated to standard protein precipitation procedures (TCA etc.); final pellet dissolved in 10.0 ml NaOH. Each tube provided 4 samples (1.0 ml each) for Lowry determination. df=40. Mean of 20 determinations = Total Protein (A).

0.1 ml homogenate placed in each of 5 tubes & volume brought to 1.0 ml. 2 samples (0.1 ml each) used for alkaline phosphatase determination. df=200 Sp Act (B)=Mean of 10: individual value/ protein A, Sp Act (C)=Mean of 20: mean OD/individual protein values.

0.1 ml homogenate placed in 1 tube & volume brought to 1.0 ml.

700 x g (10 min)

Super / Ppt

4 samples (0.1 ml) from each of super and ppt used for alkaline phosphatase determinations. df=200.

Super Sp Act (D) n=4
Ppt Sp Act (E) n=4
Mean Sp Act (F) n=8,

all using protein value A.

0.5 ml homogenate placed in 1 tube & volume brought to 5.0 ml. 10 samples (0.1 ml) used for alkaline phosphatase determinations. df=200. Sp Act (G)=Mean of 10: individual value/ protein value A, Sp Act (H)=Mean of 20: mean OD/individual protein values.

Calculations of specific activity B & G account for variation due to obtained alkaline phosphatase OD, and C & G account for variation due to obtained protein OD.

Table A-2 Variation in Total Duodenal Protein and Alkaline Phosphatase Specific Activity Due to Biochemical Methods I

1. TOTAL DUODENAL PROTEIN

GROUP	PROTEIN CONTENT (mg) ± SE	SE AS % OF MEAN	N
A	19.04 ± 0.3	1.5	20

2. ALKALINE PHOSPHATASE SPECIFIC ACTIVITY

GROUP	ALKALINE PHOSPHATASE SP ACT (μg/hr/mg)	SE AS % OF MEAN	N
B	860.6 ± 18.1	2.1	10
C	861.0 ± 11.9	1.4	20
D	617.3 ± 12.1	2.0	4
E	1104.5 ± 47.2	4.3	4
F	858.5 ± 93.5	10.9	8
G	887.1 ± 38.8	4.4	10
H	891.1 ± 12.6	1.4	20

Refer to Table 3 for explanation of group composition and specific activity calculation.

No significant differences using Student t test between any 2 of: B, C, F, G, H (90%).
F not significantly different from D or E (90%).
D and E significantly different (1%).
D significantly different from B, C, G, H (5%).
E not significantly different from B, C, G, H (5%).

Table A-3 Variation in Total Duodenal Protein and Alkaline Phosphatase Specific Activity Due to Biochemical Methods II

One small duodenum (from culture material) lyophilized then ground in distilled water; volume brought to 2.0 ml.

0.5 ml homogenate placed in one tube & treated to standard protein procedure; final pellet dissolved in 10 ml NaOH. 10 samples (1.0 ml) used for Lowry assay. df=40. n=10.

TOTAL PROTEIN: 2.3 ± 0.01 (mg) ± SE

SE as % MEAN: 0.4%

0.1 ml homogenate used undiluted in each of 10 alkaline phosphatese assays. df=20. n=10.

ALKALINE PHOSPHATASE: 44.8 ± 2.0 SP ACT (µg/hr/mg)

SE AS % MEAN: 3.4%

2.3 used as protein value in sp act calculation

N.B. In both assays all samples were from one tube thus variation would be due mainly to assay rather than preparative methods.

APPENDIX V

MATERIALS AND METHODS: DUNCAN'S NEW MULTIPLE RANGE COMPARISON

Data were analysed using Duncan's New Multiple Range Comparison (Duncan 1955, 1957). A Fortran program developed for this test by D.L. Mallon (see Goldberg 1971) was modified by C.S. Shay and entered on tape as DUNCAN (n=20) and D (n=26) using Fortran IV. In this test the variance ratio (F) is first calculated. If it is significant then each treatment mean is compared with every other treatment mean using the least significant difference (lsd) to determine which groups are significantly different from each other. As the number of groups compared is increased the test becomes more powerful i.e. is more likely to detect real differences (Kirk 1968).

The programme is found on the following pages.

```
!FLAG DUNCAN
 FLAG VERSION D04
 WITH>LS

0001.000   0001                DIMENSION WORK(20),P(20),N1(20),X(200),
0002.000   0002            1   SUM(20),SUMS(20),AVG(20),SSUM(20),SOS(20),L
SR(20),
0003.000   0003            1   CAL(200),ACT(200),DEV(20)
0004.000   0004                REAL LSR,MSQ1,MSQ2
0005.000   0005                INTEGER WORK,P
0006.000   0006                DATA WORK/' A  ',' B  ',' C  ',' D  ',' E
',
0006.500   0007            1   ' F',
0007.000   0008            1   'G  ',' H  ',' I  ',' J  ',' K  ',' L  ','
M  ',
0008.000   0009            1   ' N  ',' O  ',' P  ',' Q  ',' R  ',' S  ','
 T'/
0009.000   0010                DATA P/' P=1',' P=2','P=3',' P=4',' P=5','
 P=6',
0010.000   0011            1   ' P=7',' P=8',' P=9','P=10','P=11','P=12',
0011.000   0012            1   'P=13','P=14','P=15','P=16','P=17','P=18',
0012.000   0013            1   'P=19','P=20'/
0013.000   0014     99         PRINT 205
0014.000   0015     205        FORMAT(1X,'ENTER NUMBER OF GROUPS')
0015.000   0016                PRINT 204
0016.000   0017     204        FORMAT(1X,'IF YOU DO NOT WISH TO CONTINUE,
0017.000   0018            1   ENTER 0.0')
0018.000   0019                READ 105,N
0019.000   0020                IF(N.LT.1.0) GO TO 60
0020.000   0021                PRINT 206
0021.000   0022     206        FORMAT(1X,'ENTER NUMBER OF INDIVIDUALS IN F
OLLOWING
0022.000   0023            1   GROUPS')
0023.000   0024                PRINT 210,(WORK(I),I=1,N)
0024.000   0025                READ 110,(N1(I),I=1,N)
```

```
0025.000   0026              DO 15 I=1,N
0026.000   0027              SUM(I)=0.
0027.000   0028     15       SUMS(I)=0.
0028.000   0029              NN=1.
0029.000   0030              NNN=0.
0030.000   0031              PRINT 211
0031.000   0032     211      FORMAT(1X,'ENTER INDIV. VALUES,8 PER LINE,
0032.000   0033           1  FOR GROUPS:')
0033.000   0034              DO 10 I=1,N
0034.000   0035              PRINT 215,WORK(I)
0035.000   0036              NNN=NN+N1(I)-1
0036.000   0037              READ 115,(X(J),J=NN,NNN)
0037.000   0038              DO 20 J=NN,NNN
0038.000   0039              SUM(I)=SUM(I)+X(J)
0039.000   0040     20       SUMS(I)=SUMS(I)+X(J)**2.
0040.000   0041              AVG(I)=SUM(I)/N1(I)
0041.000   0042              SSUM(I)=SUM(I)**2./N1(I)
0042.000   0043              SOS(I)=SUMS(I)-SSUM(I)
0043.000   0044              DEV(I)=SQRT(SOS(I)/(N1(I)-1))
0044.000   0045              PRINT 300,AVG(I),DEV(I)
0045.000   0046     300      FORMAT(1X,'AVG=',F10.3,'STD.DEV.=',F10.3)
0046.000   0047     10       NN=NNN+1
0047.000   0048              TT=0.
0048.000   0049              TN1=0.
0049.000   0050              TS=0.
0050.000   0051              WIT=0.
0051.000   0052              DO 30 I=1,N
0052.000   0053              TT=TT+SUM(I)
0053.000   0054              TN1=TN1+N1(I)
0054.000   0055              TS=TS+SUMS(I)
0055.000   0056     30       WIT=WIT+SOS(I)
0056.000   0057              TSS=TT**2./TN1
0057.000   0058              TOT=TS-TSS
0058.000   0059              BET=TOT-WIT
0059.000   0060              DFT=TN1-1
```

```
0060.000   0061              DFB=N-1
0061.000   0062              DFW=DFT-DFB
0062.000   0063              MSQ1=WIT/DFW
0063.000   0064              MSQ2=BET/DFB
0064.000   0065              F=MSQ2/MSQ1
0065.000   0066              IF(MSQ1.GT.MSQ2) F=1./F
0066.000   0067     40       PRINT 220,F
0067.000   0068     220      FORMAT(1X,'F=',G14.7)
0068.000   0069              PRINT 230
0069.000   0070     230      FORMAT(1X,'ENTER VALUE OF F FROM TABLE FOR:
')
0070.000   0071              PRINT 225,DFB,DFW
0071.000   0072     225      FORMAT(1X,'N1=',I4,'N2=',I4)
0072.000   0073              PRINT 226
0073.000   0074     226      FORMAT(1X,'IF YOU DO NOT WISH TO CONTINUE,
0074.000   0075           1  ENTER 100.0')
0075.000   0076              READ 105,F0
0076.000   0077              IF(F.LE.F0) GO TO 50
0077.000   0078              NA=N-1.
0078.000   0079              S=SQRT(MSQ1)
0079.000   0080              PRINT 235
0080.000   0081     235      FORMAT(1X,'ENTER L.S.R. VALUES FROM TABLE F
OR:')
0081.000   0082              PRINT 240,DFW
0082.000   0083     240      FORMAT(1X,'N2=',I4)
0083.000   0084              PRINT 210,(P(I),I=2,N)
0084.000   0085              READ 110, (LSR(I),I=1,NA)
0085.000   0086              M=0.
0086.000   0087              DO 8 I=1,NA
0087.000   0088              K=I+1
0088.000   0089              NI=N-I
0089.000   0090              DO 8 J=1,NI
0090.000   0091              M=M+1.
```

```
0091.000   0092              CAL(M)=LSR(J)*S*SQRT(0.5*(1./N1(I)+1./N1(K)
))
0092.000   0093     8        K=K+1.
0093.000   0094              M=0.
0094.000   0095              PRINT 245
0095.000   0096     245      FORMAT(1X,'THE FOLLOWING GROUPS ARE NOT DIF
FERENT')
0096.000   0097              DO 7 I=1,NA
0097.000   0098              II=I+1
0098.000   0099              DO 7 J=II,N
0099.000   0100              M=M+1
0100.000   0101              ACT(M)=ABS(AVG(J)-AVG(I))
0101.000   0102              IF(ACT(M).GT.CAL(M)) GO TO 7
0102.000   0103              PRINT 250,WORK(I),WORK(J)
0103.000   0104     7        CONTINUE
0104.000   0105              DO 2 I=1,M
0105.000   0106              PRINT 255,CAL(I),ACT(I)
0106.000   0107     255      FORMAT(1X,2F10.3)
0107.000   0108     2        CONTINUE
0108.000   0109              GO TO 40
0109.000   0110     105      FORMAT (G.0)
0110.000   0111     110      FORMAT (20G.0)
0111.000   0112     115      FORMAT (8G.0)
0112.000   0113     210      FORMAT (20A4)
0113.000   0114     215      FORMAT (A4)
0114.000   0115     250      FORMAT (10X,2A4)
0115.000   0116     50       GO TO 99
0116.000   0117     60       END
```

APPENDIX VI

RESULTS: DUODENAL WEIGHT FOLLOWING EXPERIMENTS PERFORMED IN VIVO

1. Fresh Weight

a. Stage

Embryos treated with L-T_4 possessed duodenal fresh weights significantly greater than did other stage 43 embryos which had been treated 24 hours previously, with the exception of the D-T_4 treated group. The latter group did not differ from the L-T_4 or any of the other stage 43 groups 24 hours after treatment (Table A-4). Stage 42 embryos which had been treated 24 hours previously did not differ from normal stage 42s when their fresh weights were compared, with the exception of the D-T_4 treated group which was significantly greater than the others (Table A-4). Stage 43 embryos which had been treated 48 hours previously (L-T_4, D-T_4, and saline-treated) had fresh weights falling between the stage 43, 24 hour D-T_4 and L-T_4 values (Table A-4). These groups were all significantly different from the stage 43, 24 hour saline-treated, and the L-T_4, 48 hour was significantly different from the normal stage 43 group.

Table A-4

Fresh Duodenal Weight - Staged Results

Time After Treatment - Stage	Treatment Weight (mg)						
	41S (4)	42S (8)	42L (5)	42S (8)	42W (11)	42N (38)	42D (8)
24 Hr - 41,42	28.6 ± 3.5	32.1 ± 1.0	34.8 ± 4.8	35.4 ± 2.3	35.4 ± 2.8	35.8 ± 0.7	43.4 ± 3.5
	S (8)	N (26)	W (20	D (10)			L (16)
24 Hr - 43	38.3 ± 1.5	41.0 ± 1.1	45.0 ± 2.6	47.2 ± 4.4			54.3 ± 2.5
				S. (7)	D (5)	L (8)	
48 Hr - 43				48.7 ± 4.0	51.2 ± 2.6	53.5 ± 5.0	
	D (19)	S (16)		W (14)	L (20)	N (43)	
48 Hr - 44	59.1 ± 2.0	60.1 ± 2.6		60.4 ± 2.6	63.7 ± 1.8	67.7 ± 2.1	
							L (10)
72 Hr - 44							69.2 ± 3.2
	W (8)	D (15)		L (16)	S (14)	N (20)	
72 Hr - 45	69.1 ± 3.5	71.6 ± 4.1		78.2 ± 3.5	78.9 ± 2.4	81.7 ± 2.9	
	L (18)						
48 Hr - 45	70.0 ± 2.8						

At 48 hours, the duodenal fresh weights of the stage 44 L-T_4 treated group did not differ significantly from those of any other stage 44, 48 hour group (Table A-4). The normal stage 44 embryos however had duodenal fresh weights which were significantly greater than those of windowed, saline, and D-T_4 treated groups. When stage 44 L-T_4 treated, 72 hour duodenal fresh weights were included in the comparison, the only significant difference which appeared was between the largest (stage 44, 72 hr, L-T_4) and smallest (stage 44, 48 hr, D-T_4) weights (Table A-4).

Seventy-two hours after treatment no significant differences were found among the stage 45 embryos with respect to their fresh duodenal weights (Table A-4).

b. Time Pool

When pooled according to time following treatment, at 24 hours the fresh weights of L-T_4 treated embryonic duodena were significantly greater than those of normal and saline-treated embryos; D-T_4 treated and windowed fresh weights were significantly greater than saline-treated weights (Table A-5). At 48 hours the normal fresh weights were significantly greater than windowed and saline-treated weights, and L-T_4 treated were significantly greater than saline and D-T_4 treated. Seventy-two hours after treatment the fresh duodenal

Table A-5

Fresh Duodenal Weight - Results Pooled According to Time

Time After Treatment (hr)	Treatment Weight (mg)				
24	S (16) 36.9 ± 1.4	N (26) 41.0 ± 1.1	W (20) 45.0 ± 2.6	D (19) 46.1 ± 2.7	L (24) 50.1 ± 2.5
48	D (26) 56.8 ± 1.7	S (24) 57.5 ± 2.5	W (14) 60.4 ± 2.6	L (46) 64.4 ± 1.8	N (43) 67.7 ± 2.1
72	W (8) 69.1 ± 3.5	D (17) 71.0 ± 3.7	L (27) 74.3 ± 2.5	S (16) 78.5 ± 2.2	N (19) 81.7 ± 2.9

weights of all time-pooled groups were statistically comparable.

2. Dry Weight

a. Stage

Twenty-four hours after treatment, the L-T_4 group's mean duodenal dry weight was significantly greater than those of all other stage 43, 24 hour groups, excepting the D-T_4 treated group (Table A-6). The dry weight of the latter group was not significantly different from the other groups. Stage 43 embryos treated 48 hours previously with L-T_4, saline, or D-T_4 did not differ from the L-T_4 and D-T_4 stage 43, 24 embryos. When the three 48 hour stage 43 groups were included in the stage 43 analysis, the dry duodenal weights of D-T_4 embryos injected 24 hours previously became significantly different from the normal stage 43 dry weight (Table A-6).

Stage 41 and 42 embryos found 24 hours after treatment had dry duodenal weights which were not significantly different from normal stage 42s, with the exception of the stage 42 D-T_4 treated group (Table A-6). The latter was not significantly different from the stage 42 saline-treated group.

Forty-eight hours after treatment the dry weights of stage 44 L-T_4 treated duodena were significantly greater than D-T_4 treated and windowed weights

Table A-6

Dry Duodenal Weight - Staged Results

Time After Treatment - Stage	Treatment Weight (mg)						
24 Hr - 41,42	41N (9) 4.3 ±0.2	42W (11) 4.4 ±0.6	41S (4) 4.9 ±0.5	42N (40) 5.3 ±0.2	42L (5) 5.3 ±0.6	42S (8) 5.6 ±0.3	42D (8) 6.6 ±0.4
24 - 43	N (26) 6.1 ±0.2	S (8) 6.5 ±0.2	W (20) 6.7 ±0.4	D (10) 7.6 ±0.7		L (16) 8.6 ±0.4	
48 - 43				D (5) 7.9 ±0.6	S (7) 8.4 ±0.7		L (8) 9.2 ±0.7
48 - 44	W (14) 8.1 ±0.5	D (19) 9.1 ±0.3	N (43) 9.5 ±0.3	S (16) 9.6 ±0.4	L (20) 10.4 ±0.4		
72 -44						L (10) 10.6 ± 0.5	
72 - 45	W (9) 11.6 ±0.5	S (14) 12.2 ±0.5	D (15) 12.3 ±0.7	N (20) 13.9 ±0.6	L (16) 14 ±1.1		
48 - 45	L (18) 11.8 ±0.5						

(Table A-6); saline-treated and normal weights were significantly greater than windowed stage 44 dry weights. Stage 44 embryos which had received L-T_4 treatments 72 hours previously had dry weights similar to stage 44 embryos examined 48 hours after treatment, and compared similarly with the other stage 44s (Table A-6).

When the dry weights of stage 45 embryos were compared 72 hours after treatment, the L-T_4 treated group was significantly different from all other groups except the stage 45 normals; the latter did not differ from the other groups (Table A-6).

b. Time Pool

At 24 hours the dry duodenal weights of the L-T_4 treated embryos remained significantly greater than all other groups except the D-T_4 treated when time-pooled results were compared (Table A-7). The windowed, saline, and normal groups did not differ from each other at this time.

Forty-eight hours following treatment, the dry weight of the time-pooled L-T_4 group was significantly greater than all others at the same time (Table A-7). Normal stage 44 duodenal dry weight was significantly greater than windowed stage 44 dry weight as found in the stage comparison; neither of these group's values changed in this comparison, being unaffected by the

Table A-7

Dry Duodenal Weight - Results Pooled According to Time

Time After Treatment (hr)	Treatment Weight (mg)				
24	S (16) 6.1 ± 0.2	N (26) 6.1 ± 0.2	W (20) 6.7 ± 0.4	D (20) 7.6 ± 0.5	L (24) 7.9 ± 0.4
48	W (14) 8.1 ± 0.5	D (26) 8.7 ± 0.3	S (24) 9.2 ± 0.4	N (43) 9.5 ± 0.3	L (46) 10.8 ± 0.3
72	W (9) 11.6 ± 0.5	S (16) 12.2 ± 0.5	D (17) 12.2 ± 0.6	L (27) 13.0 ± 0.8	N (20) 13.9 ± 0.6

pooling of data. Seventy-two hours following treatment no significant differences occurred among the dry weights of any of the time-pooled groups.

3. Comparison of Fresh and Dry Weights

When the mean fresh and dry duodenal weights in each group of staged embryos were compared, dry weight was found to comprise a mean of 15.7% of fresh weight (Table A-8). This proportion appeared to be fairly constant, without showing any definite trend of changing with treatment, stage or time. Comparisons of individual values from various stages indicated that the proportions derived from the means did not mask variation. Therefore fresh and dry weight generally increased and decreased in tandem .

Table A-8 Relationship Between Fresh and Dry Duodenal Weight

Stage	Dry Weight as % Fresh Weight Treatment					
	N	W	S	D	L	Mean
41	13.4	-	17.1	-	-	15.2
42	15.1	12.4	15.8	15.2	15.2	14.7
43	14.9	14.9	17.0 (24) 17.0 (48)	16.1 (24) 15.4 (48)	15.8 (24) 17.2 (48)	16.1
44	14.0	13.4	16.0	15.4	16.3 (48) 15.3 (72)	15.1
45	17.0	16.8	15.5	17.2	16.8 (48) 18.0 (72)	16.9

Mean of all values = 15.7
Time after treatment is indicated in parentheses where one stage/treatment group was found at more than one time.

APPENDIX VII

RESULTS: BODY WEIGHT FOLLOWING EXPERIMENTS PERFORMED IN VIVO

1. Stage

Twenty-four hours after treatment, the heaviest body weights were exhibited by the L-T_4 treated embryos (Table A-9). This group was significantly different from all other stage 43 groups with the exception of D-T_4 treated embryos. Stage 42 embryos encountered at this time had body weights comparable to those of normal stage 42 embryos. Stage 43 embryos, treated 48 hours previously with L-T_4, weighed more than stage 43s 24 hours after L-T_4 treatment. Both groups demonstrated similar statistical relationships to the other stage 43 groups (Table A-9).

At 48 and 72 hours, the body weights of normal stage 44 and 45 embryos differed significantly from all other groups of their respective stages (Table A-9). At 48 hours the stage 44 saline and L-T_4 treated weights were also significantly larger than the stage 44 windowed body weights. Stage 44 embryos which had been treated 72 hours previously with L-T_4 had body weights significantly greater than all other stage 44 weights.

Table A-9

Body Weight - Staged Results

Time After Treatment - Stage	Treatment Weight (mg)					
24 - 41,42	41S (4) 13.6 ± 0.5	41N (9) 13.8 ± 0.5	42N (29) 15.0 ± 0.3	42S (5) 16.2 ± 0.5	42D (7) 16.8 ± 0.4	
24 - 43	S (8) 16.4 ± 0.6	N (17) 16.7 ± 0.3	W (16) 17.2 ± 0.4	D (10) 18.0 ± 0.7	L (15) 18.9 ± 0.4	
48 - 43	S (6) 16.4 ± 1.0	D (5) 17.1 ± 0.5				L (8) 19.2 ± 1.0
48 - 44	W (12) 18.8 ± 0.5	D (19) 19.8 ± 0.3	L (20) 20.7 ± 0.4	S (16) 21.1 ± 0.5	N (31) 22.6 ± 0.4	
72 - 44						L (10) 24.8 ± 1.0
72 - 45	W (8) 22.2 ± 0.8	D (15) 22.6 ± 0.6	S (14) 23.3 ± 0.8	L (16) 24.2 ± 1.0	N (20) 27.1 ± 0.4	
48 - 45		L (18) 22.6 ± 0.7				

2. Time Pool

When the body weight data were pooled according to time, at twenty-four hours the weight of the L-T_4 treated group remained the largest, significantly greater than all groups except the D-T_4 treated (Table A-10). The mean body weight of the latter group was significantly larger than that of the saline-treated group (24 hr).

At 48 hours, the body weight derived from pooled L-T_4 treated values was significantly larger than all other pooled weights (Table A-10). Normal embryos (stage 44) differed only from saline-treated and windowed embryos in this comparison. When time-pooled data were compared at 72 hours, the normal embryos were of significantly larger body weight than all other groups as in the staged comparison (Table A-10).

Table A-10

Body Weight - Results Pooled According to Time

Time After Treatment (hr)	Treatment Weight (mg)				
24	S (16) 16.3 ± 0.3	N (17) 16.7 ± 0.3	W (13) 17.2 ± 0.4	D (17) 17.5 ± 0.5	L (16) 18.6 ± 0.5
48	W (12) 18.8 ± 0.5	D (26) 18.9 ± 0.4	S (23) 20.1 ± 0.8	N (46) 21.2 ± 0.4	L (31) 22.6 ± 0.4
72	W (8) 22.2 ± 0.8	D (17) 22.5 ± 0.6	S (16) 23.3 ± 0.7	L (27) 24.3 ± 0.7	N (20) 27.1 ± 0.4

APPENDIX VIII

RESULTS: DETERMINATIONS OF ALKALINE PHOSPHATASE SPECIFIC ACTIVITY, PROTEIN CONTENT, AND WEIGHT OF NORMAL DUODENAL QUARTERS

Determinations of alkaline phosphatase specific activity, protein content, and fresh and dry weight were performed on normal duodenal quarters to determine the variation inherent among them for comparison with the experiments performed *in vitro*. The quarters were arbitrarily designated by the letters A to D, proceeding from the proximal to distal segment. Determinations were done on quarters from embryos of stages 42 to 45 and the results obtained at each stage were then compared statistically using Duncan's New Multiple Range Comparison (Duncan, 1955, 1957).

1. Protein Content

Comparison of the total protein contents of normal quarters revealed no significant differences among any of the four quarters at any stage, 42 through 45 (Table A-11). No particular quarter tended to exhibit a consistently high or low value. There was a slight tendency towards increase in protein content with increasing stage, with the exception of stage 44.

Table A-11 Total Protein Content, Alkaline Phosphatase Specific Activity, and Fresh and Dry Weights of Duodenal Quarters From Normal Embryos. I. Stages 42 and 43.

	Duodenal Quarter				
	A	B	C	D	Mean
Stage 42 (7)					(28)
Tot Prot (mg)	0.85 ± 0.08	0.75 ± 0.08	0.74 ± 0.09	0.93 ± 0.30	0.82 ± 0.08
Sp Act (μg/hr/mg)	454.4 ± 45.5	393.9 ± 46.7	316.5 ± 45.5	318.9 ± 45.0	370.9 ± 24.2
F Wt (mg)	9.9 ± 0.4	9.0 ± 0.8	8.8 ± 0.7	8.0 ± 0.3	8.9 ± 0.3
D Wt (mg)	1.6 ± 0.08	1.5 ± 0.09	1.4 ± 0.1	1.3 ± 0.04	1.5 ± 0.4
D Wt/F Wt (%)	16.2	16.7	15.9	16.2	16.5
Stage 43 (8)					(32)
Tot Prot (mg)	0.85 ± 0.1	0.86 ± 0.09	1.1 ± 0.2	0.76 ± 0.07	0.89 ± 0.07
Sp Act (μg/hr/mg)	885.3 ± 313.0	843.4 ± 248.0	740.9 ± 278.4	844.5 ± 266.8	828.5 ± 132.3
F Wt (mg)	10.2 ± 0.5	10.4 ± 0.3	10.1 ± 0.7	8.8 ± 0.6	9.9 ± 0.3
D Wt (mg)	1.6 ± 0.1	1.8 ± 0.09	1.5 ± 0.1	1.4 ± 0.06	1.6 ± 0.05
D Wt/F Wt (%)	15.7	17.3	14.8	15.9	16.1

Table A-11 Continued

II. Stages 44 and 45

	Duodenal Quarter				
	A	B	C	D	Mean
Stage 44 (10)					(40)
Tot Prot (mg)	0.63 ± 0.08	0.76 ± 0.07	0.66 ± 0.06	0.67 ± 0.05	0.68 ± 0.03
Sp Act (μg/hr/mg)	2466.7 ± 381.8	1804.3 ± 358.0	1711.0 ± 264.1	1896.6 ± 337.7	1896.6 ± 169.2
F Wt (mg)	14.2 ± 0.8	13.9 ± 0.9	11.7 ± 1.0	12.4 ± 0.6	13.1 ± 0.4
D Wt (mg)	2.3 ± 0.1	2.9 ± 0.4	2.0 ± 0.2	2.1 ± 1.0	2.3 ± 0.1 (36)
D Wt/F Wt (%)	16.2	20.9	17.1	16.9	17.1 (36)
Stage 45 (5)					(20)
Tot Prot (mg)	0.93 ± 0.2	0.94 ± 0.1	0.87 ± 0.2	0.88 ± 0.2	0.91 ± 0.08
Sp Act (μg/hr/mg)	5207.3 ± 966.5	4650.6 ± 885.3	4727.0 ± 878.8	4626.3 ± 991.5	4802.8 ± 383.6
F Wt (mg)	18.1 ± 2.6	16.9 ± 1.6	15.0 ± 1.7	14.7 ± 1.5	16.2 ± 1.1
D Wt (mg)	2.9 ± 0.4	2.7 ± 0.2	2.6 ± 0.2	2.6 ± 0.3	2.7 ± 0.15
D Wt/F Wt (%)	16.0	16.0	17.3	17.7	16.9

Significant Differences ($p < .05$) were found in dry weights of quarters at stage 43: D C A B using Duncan's New Multiple Range comparison. No significant differences were found in the remaining data when quarters of the same stage were compared (protein content, alkaline phosphatase specific activity, fresh weight and dry weight). Means were determined using values from all quarters at each stage.

Summing the mean values obtained for all quarters at each stage produced estimated total duodenal protein contents of: 3.3 mg, stage 42; 3.5 mg, stage 43; 2.7 mg, stage 44; and 3.6 mg, stage 45. Comparison of these estimated values with those obtained for intact duodena in the experiments performed in vivo (Table 7) shows that the estimated values all approximate those obtained for stage 43 to 44 control duodena. Therefore, the division of duodena into quarters may have resulted in the loss of some protein, as shown by the lower than normal estimated protein contents for stages 44 and 45.

2. Alkaline Phosphatase Specific Activity

When the alkaline phosphatase specific activity of normal duodenal quarters was measured, no significant differences were found anong the quarters at any one stage examined (Table A-11). A tendency (not significant) was seen for the proximal segment (A) to possess the greatest specific activity at all stages, particularly stages 44 and 45. Specific activity increased with each progressessive stage, the largest increase occurring from stage 44 to 45 (Table A-11). Compared to the specific activities of duodena from experiments performed in vivo (Table 6), stage 42 quarter duodena showed specific activities approximating those shown by stage 41 intact duodena. (Stage 41 and 42 normal and saline-treated

specific activities were not significantly different in intact duodena.) The specific activities of stage 43 quarters (Table A-11) were similar to those of stage 42 intact values (Table 6), and the latter were not significantly different from those of intact control stage 43 duodena. The specific activities calculated for stage 44 and 45 duodenal quarters (Table A-11) were similar to those of intact stage 44 control duodena, and intermediate between stage 45 control and L-T_4 treated values respectively (Table 6).

Therefore, duodenal quarters did not differ significantly in alkaline phosphatase specific activity, although a trend for the greatest activity to occur in the proximal segment was seen. The process of quartering the duodena did not appear to greatly affect specific activity, although the activities of stages 42 and 43 were slightly lower, and of stage 45 were slightly higher than the comparable intact activities.

3. Fresh Weight

When the fresh weights of duodenal quarters were compared, no significant differences were found among them at any stage examined (Table A-11). A general tendency was observed for fresh weight to decrease proceeding from the proximal to distal segment (A to D). Exceptions to this trend were that the fresh

weights of segments A and B, and C and D, were 'reversed' at stages 42 and 44 respectively (Table A-11). The mean fresh weights of all quarters increased from stages 42 to 45 (Table A-11).

Summing the mean values for all four quarters at each stage produced estimated values for the fresh weights of entire duodena of: 37.5 mg, stage 42; 39.5 mg, stage 43; 52.2 mg, stage 44; and 64.7 mg, stage 45. Comparison with those of intact duodena from the experiments *in vivo* (Table A-4) showed that estimated stage 42 and 43 fresh weights approximated the intact weights of the corresponding stages. The fresh weights estimated from stage 44 and 45 quarters were both slightly lower than the corresponding intact values. Therefore, the division of duodena into quarters may have produced a slight decrease in fresh weight at the later stages, compared to intact duodena.

4. Dry Weight

The dry weights of duodenal quarters, obtained from normal embryos, increased from stages 42 to 45 (Table A-11). Comparison of the dry weights of the four quarters at each stage revealed a significant difference at stage 43: the B segment weighed significantly more than the D and C segments, but did not differ significantly in dry weight from the A segment; the dry weights of

segments A, D, and C did not differ significantly from each other (Table A-11). In comparisons of dry weights at the other stages, no significant differences were found among the quarters. When compared within each stage, all four quarters were quite similar in dry weight (Table A-11), and the significant differences encountered at stage 43 result from the comparatively small Standard Errors of the Mean occurring in the stage 43 weights.

The following estimated dry weights for entire duodena were obtained by summing the means of all quarters at each stage: 5.8 mg, stage 42; 6.3 mg, stage 43; 9.3 mg, stage 44; and 10.8 mg, stage 45. These values reflect the increase in dry weight with increasing stage shown by the quarters, and compare well with the dry weights obtained for intact duodena in the experiments performed *in vivo* (Table A-6). The exception was that stage 45 estimated dry weights were slightly lower than the intact control stage 45 weights. Therefore, the division of normal duodena into quarters prior to dry weight determination did not greatly effect the resultant dry weights except for a slight decrease in weight at stage 45.

5. Relationship Between Fresh and Dry Weight

Comparison of fresh and dry weights of duodenal quarters was performed by dividing dry weight by fresh weight (result expressed as a percentage). The mean

of all such determinations (i.e. the mean of all quarters and stages, (Table A-11) was 16.7%. This was not very different from the mean of 15.7% determined using intact duodena (Table A-8). As in the results of the experiments performed *in vivo*, no pattern of change in this relationship could be observed which could be correlated with stage or quarter. Therefore, the relationship between duodenal fresh and dry weight was apparently little changed by the division of duodena into quarters.

APPENDIX IX

RESULTS: DUODENA CULTURED 24 HOURS *IN VITRO*

Duodenal explants cultured *in vitro* for 24 hr were all explanted as whole (undivided) duodena.

1. Total Protein Content

Following 24 hr of culture *in vitro*, total protein content was significantly higher in explanted duodena cultured in the presence of media containing L-T_4 or D-T_4 (5.5 ng/ml) than in those cultured with normal medium, i.e. without hormone (Table A-12). The latter group's mean protein content was less (not significant) than that of stage 41 (whole) explants also cultured 24 hr in normal media. The stage 41 explants likewise had protein contents significantly less than those of stage 42 explants cultured with L- or D-T_4. No significant differences in protein content were observed among stage 43 duodenal explants cultured 24 hr *in vitro* (Table A-12).

In comparison with the experiments performed *in vivo*, the preceding results approximate the protein contents determined for stage 41 to 43 duodena (Table 4). The normal protein contents of these stages did not differ significantly, and the value determined using explants

Table A-12 Total Protein Content, Alkaline Phosphatase Specific Activity, Fresh and Dry Weights of Duodenal Explants Cultured 24 Hours *In Vitro*

	Stage 41 - 42				Stage 43		
	Treatment						
Alkaline Phosphatase Sp Act (µg/hr/mg)	41N 372.5 24.0	42D 702.2 ± 44.8	42N 902.3 ± 114.1	42L 909.6 ± 65.2 *	43N 742.8 ± 148.8	43D 1008.9 ± 123.1	43L 1074.1 ± 123.1
Total Protein (mg)	42N (9) 1.34 ± 0.14	41N (13) 1.5 ± 0.1	42L (11) 1.89 ± 0.09	42D (10) 1.97 ± 0.11	43D (9) 2.03 ± 0.19	43L (7) 2.21 ± 0.24	43N (8) 2.24 ± 0.14
Fresh Weight Before Culture (mg)	42N 35.6 ± 1.5	42D 38.8 ± 2.2	42L 40.9 ± 1.14		43D (2) 46.3	43N (4) 49.3	43L (1) 50.5 ± 3.4
Fresh Weight After Culture (mg)	42N 31.6 ± 1.4	42L 33.7 ± 1.2	42D 35.0 ± 1.9		43N 36.9 ± 2.0	43D 38.5 ± 2.2	43L 41.7 ± 2.3
Dry Weight (mg)	42N 4.4 ± 0.22	42L 4.63 ± 0.13	42D 4.8 ± 0.19	41N 5.0 ± 0.2	43N 4.3 ± 0.21	43D 5.1 ± 0.26	43L 5.5 ± 0.03
Dry Wt/Fresh Wt AC (%)	42D 13.7	42L 13.7	42N 13.9		43N 11.6	43D 13.2	43L 13.2

Changes in fresh weight before and after culture were not significantly different (student's t test, $p > 0.05$).
Stages 42 and 43 were compared separately (Duncan's).

* When stage 41 was omitted from comparison, stage 42 alkaline phosphatase sp. act. were not significantly different (NSD).

cultured 24 hr with normal media was similar to that of stage 41 saline-treated duodena (which did have protein contents significantly less than stage 42 normal duodena). Therefore it is difficult to determine whether the difference in protein content in the stage 42 cultures was due to decrease in protein content in the normal cultures, or increase or maintenance in the cultures containing hormone.

2. Alkaline Phosphatase Specific Activity

After 24 hours in culture, no significant differences in alkaline phosphatase specific activity were observed among duodena explanted at stage 42 (Table A-12). However, if stage 41 results were included in the statistical comparison with the stage 42 results, the specific activity of the cultures with D-T_4 became significantly different from those of the other stage 42 treatment groups. No significant differences in phosphatase activity were observed among the cultures of stage 43 explants.

The alkaline phosphatase specific activities determined after 24 hr of culture all approximated values of similar stage to the stages of explant, when comparison was made with results of experiments performed *in vivo* (Table 6). Therefore, alkaline phosphatase activity neither increased nor decreased markedly in these cultures.

3. Fresh Weight

The fresh weights of stage 42 and 43 duodenal explants were determined both before and after 24 hr of culture. Initially there were no significant differences in fresh weight (Table A-12), and following 24 hr of culture, none had appeared among any of the groups, within cultures of either stage. In all cases, mean duodenal fresh weights had decreased following culture compared to their initial fresh weights. The mean change in weight before and after culture was smaller in stage 42 than stage 43 cultures (5 and 9.7 mg respectively). (The number of comparisons made using stage 43 cultures was comparatively small.) Stage 42 explants cultured with medium containing L-T_4 showed a larger decrease in weight than did other stage 42 cultures. In all cases, stage 42 and 43, the differences in fresh weight before and after culture were not significant (Student's t test).

Comparison with fresh duodenal weights determined for the experiments performed *in vivo* (Table A-4), revealed the fresh weights before and after culture to be within the normal ranges for stages 42 and 43.

4. Dry Weight

Stage 43 whole duodenal explants had similar dry weights after 24 hr of culture, regardless of the medium used (Table A-12). Duodena from stage 41 embryos had slightly larger dry weights following culture for the same length of time (Table A-12). When the dry

weights of stage 43 explants were compared, those cultured with L-T_4 or D-T_4 in the media had significantly greater dry weights than those cultured with normal medium (Table A-12).

When the relationships between fresh (after culture) and dry weights were compared, values of 13.8 and 12.7% determined for stages 42 and 43 respectively (dry weight/fresh weight after culture, expressed as percentage). These values were lower than those determined for the experiments performed in vivo (Table A-6), indicating that following culture, duodenal dry weight had decreased in proportion to fresh weight. Stage 42 duodena which had been cultured in vitro for 24 hr had dry weights similar to those found for normal stage 42 duodena (Table A-6). The dry weights determined for all stage 43 cultures were less than the normal stage 43 dry weights (Table A-6). Therefore duodenal dry weight appears to have decreased in relation to fresh weight following culture. This change may be due to failure of dry weight to increase (stage 42), or to decrease in dry weight (stage 43) during culture.

APPENDIX X

RESULTS: DUODENAL QUARTERS CULTURED 48 HOURS *IN VITRO*

1. Stage 42

a. Fresh Weight

At the beginning of the 48 hour culture period, stage 42 duodenal quarters which were to be cultured with 14 ng L-T_4 /ml of medium had significantly smaller fresh weights than did those which formed the group cultured with 14 ng L-T_4 /100 ml (Table A-13). This initial difference was unintentionally produced by random placement of quarters into groups. Similarly, following culture, the mean fresh weight for this group was significantly smaller than for those cultured with L-T_4 (14 ng/100 ml) or with D-T_4 (Table A-13). The initial significant difference in fresh weight indicates that the difference between the two L-T_4 groups following culture was probably not produced by the conditions of culture. The fresh weights of the other groups cultured without BSA did not differ significantly from each other, either before or after culture (Table A-13). When the fresh weights before and after culture were compared (Student's t test), the two weights differed significantly in normal and L-T_4 (14 ng/100 ml) treated explants.

Table A-13

Fresh and Dry Weights of Stage 42 Duodenal Quarters Cultured 48 Hours In Vitro

	No BSA				With BSA			
Fresh Wt Before (mg)	L(14/1) (16) 6.9 ± 0.3	N (24) 8.1 ± 0.3	D (9) 8.3 ± 0.6	L(14/100) (27) 8.4 ± 0.3	L(14/100) (21) 8.1 ± 0.4	D (22) 8.2 ± 0.3	N (23) 8.7 ± 0.4	L(14/1) (22) 8.8 ± 0.3
Fresh Wt After (mg)	L(14/1) 5.7 ± 0.3	N 6.4 ± 0.2	L(14/100) 7.0 ± 0.3	D 7.6 ± 0.6	L(14/100) 6.1 ± 0.05	D 6.4 ± 0.2	L(14/1) 6.9 ± 0.3	N 7.0 ± 0.3
Dry Wt (mg)	N 0.67 ± 0.06	L(14/1) 0.69 ± 0.05	L(14/100) 0.89 ± 0.02	D 1.06 ± 0.09	L(14/100) 0.75 ± 0.09	L(14/1) 0.79 ± 0.06	D 0.84 ± 0.04	N 0.90 ± 0.03
D Wt/F Wt Ac (%)	N 10.5	L(14/1) 12.1	L(14/100) 12.7	D 13.1	L(14/100) 12.3	L(14/1) 11.4	D 13.1	N 12.8
Change in F Wt-t	SD	NSD	SD	NSD	SD	NSD	NSD	NSD

The lines underscoring the data indicate the results of Duncan's comparisons treating cultures with and without BSA separately.
D Wt/F Wt AC (%) is measure of relationship of dry weight to fresh weight (after culture).
Results of Student's t tests comparing fresh weights before and after culture are shown as significant (SD) or not significant (NSD) at p=.05.

In cultures with BSA added to the media, the fresh weights of all groups were similar to each other when compared either before or after culture (Table A-13). All groups, except that cultured with 14 ng L-T_4/ml + BSA, showed significant changes in fresh weight before and after culture (t test). When all groups, with and without BSA, were compared in the same Duncan's analysis, the fresh weight before culture with 14 ng L-T_4/ml was found to have been significantly smaller than in similar cultures with BSA (Table A-14). No relationships found in separate comparisons of groups with and without BSA were altered in the combined comparison.

The fresh weights of stage 42 duodenal quarters prior to culture were all similar to weights determined for similar quarters of the same stage in Appendix VIII (Table A-11), with the exception of quarters cultured with 14 ng L-T_4/ml of medium, which had smaller fresh weights. Estimation of total duodenal fresh weight (by multiplication of quarters' weights by four) gave a value closer to the stage 41 than 42 normal fresh weights for the aforementioned group (Table A-4). The fresh weights of all groups had decreased when they were determined following culture, therefore duodenal fresh weight did not increase during culture <u>in vitro</u> as in intact duodena during this 48 hr period.

Table A-14 Results of Duncan's New Multiple Range Analysis Including Weight Data from Stage 42, 48 Hour Cultures With and Without BSA in the Same Comparison

Fresh Weight Before Culture (mg)

L(14/1) N L(14/100)+BSA D+BSA D L(14/100) N+BSA L(14/100)+BSA

Fresh Weight After Culture (mg)

L(14/1) L(14/100)+BSA D+BSA N N+BSA L(14/1)+BSA L(14/100) D

Dry Weight (mg)

N L(14/1) L(14/100)+BSA L(14/1)+BSA D+BSA L(14/100) N+BSA D

The data used in these comparisons is found in Table A-13.

b. Dry Weight

Following culture for 48 hr in medium which did not contain BSA, D-T_4 treated stage 42 explants had significantly greater dry weights than did explants cultured with either 14 ng L-T_4 /ml or normal media (Table A-13). The dry weights of all of these groups did not differ significantly from the mean dry weight of explants cultured with 14 ng L-T_4 /100 ml of media. All explants cultured in media containing BSA had statistically similar dry weights (Table A-13), and no changes in any of the above relationships appeared when all groups were included in the same Duncan's New Multiple Range comparison (Table A-14).

The dry weights observed after stage 42 explants had been cultured for 48 hr were smaller than those of normal stage 42 duodenal quarters (Table A-11). Multiplication by four, to estimate whole duodenal dry weights, gave values less than those obtained for normal, whole stage 41 duodena (Table A-6). Therefore the dry weights of duodenal quarters appear to have decreased when maintained *in vitro* for 48 hr.

The relationship between fresh and dry weight was determined for each group (dry weight/fresh weight after culture, expressed as percentage). For all stage 42 cultures, the mean relationship was 12.3% (Table A-13). In cultures with and without BSA, the highest values were

calculated for quarters cultured with D-T_4 in the media. These values were all less than those calculated for duodena which had not been cultured (Tables A-8 and A-11) indicating that dry weight decreased relative to fresh weight following 48 hours in culture.

2. Stage 43

a. Total Protein Content (Quarters)

The largest mean protein content following culture of stage 43 quarters for 48 hr was found in explants cultured with D-T_4 (Table A-15), as in the stage 42 (48 hr) cultures (Table 11). However, no significant differences in protein content appeared when the results, with and/or without BSA, were compared. The protein contents of these cultures were similar to those found after 48 hr of culture using stage 42 duodenal quarters, and therefore were also less than normal (uncultured) protein levels.

b. Alkaline Phosphatase Specific Activity

In stage 43 experiments, alkaline phosphatase specific activity was highest in explants cultured with 14 ng L-T_4 /ml of medium with or without BSA (Table A-15). However, no significant differences in specific activity appeared in comparisons within and between the two sets of results (with and without BSA). The mean specific

Table A-15 Protein Content, Alkaline Phosphatase Specific Activity, and Fresh and Dry Weights of Stage 43 Duodenal Quarters Cultured 48 Hours *In Vitro*

	No BSA			With BSA		
Alkaline Phosphatase Sp Act (μg/hr/mg)	D (8) 990.3 ± 142.4	L(14/1) (11) 1060.7 ± 130.2	N (9) 1206.6 ± 139.2	N (6) 1017.9 ± 162.7	L(14/100) (7) 1299.2 ± 184.1	L(14/1) (6) 1655.7 ± 139.1
Total Protein (mg)	L(14/1) 0.35 ± 0.03	N 0.37 ± 0.04	D 0.38 ± 0.03	L(14/1) 0.22 ± 0.01	L(14/100) 0.26 ± 0.07	N 0.35 ± 0.06
Fresh Weight Before (mg)	L(14/1) 9.8 ± 0.5	N 10.0 ± 0.7	D 10.6 ± 0.8	L(14/1) 10.0 ± 0.7	N 11.0 ± 1.0	L(14/100) 11.9 ± 0.7
Fresh Weight After (mg)	L(14/1) 7.7 ± 0.4	D 7.8 ± 0.5	N 8.1 ± 0.7	L(14/100) 6.7 ± 1.0	N 8.9 ± 0.8	L(14/1) 9.6 ± 1.4
Dry Weight (mg)	N (3) 0.93 ± 0.08	D(5) 1.06 ± 0.10	L(14/1) (8) 1.10 ± 0.07	L(14/1) (7) 0.75 ± 0.10	L(14/100) (5) 1.06 ± 0.20	N(6) 1.25 ± 0.16
D Wt/F Wt AC (%)	N 11.5	D 13.6	L(14/1) 14.3	L(14/1) 7.8	L(14/100) 15.8	N 14.0
Change in F Wt	NSD	SD	SD	NSD	SD	NSD

No significant differences were found in any groups in separate or combined (no BSA and BSA) Duncan's comparisons.

activity of explants cultured with 14 ng L-T_4 /ml + BSA was almost significantly greater than that of explants cultured in normal media containing BSA (Student's t test). The phosphatase activity of the single D-T_4 treated group (no BSA) was similar to the activities of the two normal groups (Table A-15).

The alkaline phosphatase activities observed for stage 43 explants cultured 48 hr were similar to those exhibited by stage 42 explants cultured 48 hr (Table 11). Thus the activities of stage 43 cultures fell within the normal stage 42 to 43 range (Table 6). Therefore, stage 43 explants neither increased nor decreased markedly in alkaline phosphatase specific activity during 48 hr of culture *in vitro*.

c. Fresh Weight

Stage 43 quarters cultured 48 hr *in vitro* showed no significant differences in fresh weight when comparisons were made either before or after culture (Table A-15). This was observed for both sets of experiments, with and without BSA. The two normal groups and the group cultured with 14 ng L-T_4 /ml + BSA did not undergo significant changes in their fresh weights before and after culture, while the remaining groups did (Student's t test).

The initial fresh weights of stage 43 quarters used in culture approximated those determined previously

for normal stage 43 duodenal quarters (Table A-11). The cultures resembled the stage 42, 48 hr ones (Table A-13) in that the normal increase in fresh weight, observed *in vivo*, did not occur.

d. Dry Weight

Stage 43 explants all had similar dry weights following 48 hr of culture with the various media (Table A-15). The dry weights of these explants were generally larger than those of stage 42 explants cultured for the same length of time. However, the stage 43 cultured dry weights were less than those of normal stage 42 duodenal quarters (Table A-11). Estimations of total duodenal dry weight (multiplication of quarter weight by four), range from less than stage 41 normal (3.0 mg, 14 ng L-T_4 /ml + BSA), to similar to stage 42 saline-treated (4.4 mg, 14 ng L-T_4 /ml, no BSA) intact duodenal weights (Table A-6). Therefore, dry weights of stage 43 quarters appear to have decreased following culture, as in the case of the stage 42, 48 hr cultures. This is supported by the calculation of a mean value of 12.8 for the relationship between fresh weight and dry weight (Table A-15), which is lower than similar values for uncultured duodena (Tables A-8 and A-11).

APPENDIX XI

RESULTS: DUODENAL QUARTERS CULTURED FOR 72 HOURS *IN VITRO*

In this section data from preliminary experiments in which stage 42 and 43 duodenal quarters were cultured for 72 hr *in vitro* is presented. The culture media used contained L-T_4 (6.27 ng/100 ml), D-T_4 (14 ng/ml), or saline vehicle. Bovine serum albumin was not added to any of the culture media.

1. Protein Content

Comparisons of protein contents of duodenal quarters, cultured for 72 hr *in vitro*, did not indicate any significant differences among the stage 42 or 43 cultures (Table A-16). The protein contents determined for explants of both stages were similar, and all were less than those determined for normal stage 42 quarters (Table A-11). Estimation of total duodenal protein content using the largest quarter value from these cultures (stage 42 D-T_4) gives a value slightly greater than that of stage 41 saline-treated intact duodena (Table 4). Therefore, protein content of duodenal quarters appears to have decreased following 72 hours of culture.

2. Alkaline Phosphatase Specific Activity

In both stage 42 and 43 cultures, homogenates of D-T_4 treated explants possessed the highest alkaline phosphatase specific activities of the three treatment groups (Table A-16). However, in neither case was the difference significant. The phosphatase activities of the 72 cultures all varied about the levels determined for normal stage 43 quarters (Table A-11), i.e. were comparable to stage 42 to 43 activities in the experiments performed *in vivo* (Table 6). Therefore, alkaline phosphatase activity did not appear to deviate greatly from the initial levels at the time of explant, following 72 hr of culture.

3. Fresh Weights

No significant differences in fresh weight were initially present in either stage 42 or 43 duodenal quarters, prior to 72 hr of culture (Table A-16). Following culture, the fresh weights of all groups had decreased, but no significant differences had appeared among any of the treatment groups at either stage (Table A-16). Comparisons of the changes in fresh weight before and after culture, within each group, indicated significant changes in weight in both the stage 42 and 43 D-T_4 treated groups (Student's t test). However, all other groups changed as much or more in fresh weight as

did the D-T_4 treated groups, but these changes were not statistically significant.

The initial fresh weights were similar to those of normal quarters from embryos of the same stages (Table A-11). Estimated total duodenal weights (quarter multiplied by four) are in the normal ranges for the corresponding stages at the start of culture, and comparable to stage 41 intact weights at the end of culture (Table A-4). Therefore duodenal quarters decreased in fresh weight when cultured for 72 hr *in vitro*.

4. Dry Weight

The culture of duodenal explants for 72 hr in L-T_4 or D-T_4 containing media did not result in any significant differences in dry weight in either stage 42 or 43 cultures (Table A-16). All dry weights were less than those of normal stage 42 quarters (Table A-11), and when multiplied by four were less than stage 41 intact dry weights (Table A-6). Therefore dry weight of duodenal quarters decreased following 72 hr of culture. This is reflected by smaller proportions of dry weight relative to fresh weight, compared to the relationships in normal quarters or whole duodena (Tables A-8, A-11, and A-16).

Table A-16 Protein Content, Alkaline Phosphatase Specific Activity, and Fresh and Dry Weights of Stage 42 and 43 Duodenal Quarters Cultured 72 Hours *In Vitro*

	Stage 42			Stage 43		
Alkaline Phosphatase Sp Act (µg/hr/mg)	L (6.27/1) (4) 634.8 ± 59.6	N (11) 691.4 ± 70.3	D (14/1) (6) 1000.3 ± 266.5	L (6.27/1) (4) 654.4 ± 84.6	N (9) 719.4 ±118.3	D (14/1) (6) 1016.2 ± 123.9
Total Protein (mg)	N 0.35 ±0.02	L 0.36 ±0.04	D 0.41 ±0.01	D 0.35 ±0.02	L 0.36 0.04	N 0.40 ±0.02
Fresh Weight Before (mg)	L 8.6 ± 0.8	N 9.7 ± 0.7	D 9.4 ± 0.4	D 9.2 ± 0.8	N 10.7 ± 0.9	L 11.4 ± 1.1
Fresh Weight After (mg)	L 6.2 ± 0.4	N 7.1 ± 0.6	D 7.2 ± 0.4	D 6.4 ± 0.3	N 7.5 ± 0.5	L 7.5 ± 0.5
Dry Weight (mg)	L 0.85 ±0.09	D 0.95 ±0.09	N 0.97 ±0.06	D 0.85 ±0.10	N 1.00 ±0.10	L 1.00 ±0.07
D Wt/F Wt (%)	L 13.7	D 13.2	N 13.7	D 13.3	N 13.3	L 13.3
Change F Wt-t	NSD	SD	NSD	SD	NSD	NSD

Separate comparisons showed NSD within groups of same stage for any criterion.

APPENDIX XII

RESULTS: DETERMINATION OF PROTEIN CONTENT AND ALKALINE PHOSPHATASE SPECIFIC ACTIVITY OF CULTURE MEDIA

Protein content and alkaline phosphatase specific activity of culture media were measured to determine:

1) whether or not they were present in the media, and

2) whether their levels increased in the media following culture of duodenal explants. Increases in these entities after culture might indicate loss of protein and phosphatase from the duodenal explants. Results of representative protein and phosphatase determinations are described here.

Control media, representing the condition before culture, consisted of McCoy's 5A without added hormone or BSA, and McCoy's 5A to which BSA and L-T_4 were added to concentrations of 2 mg/ml and 14 ng/ml respectively. These media were from stocks of regular culture media used for the experiments *in vitro*. Culture media consisted of McCoy's 5A, to which had been added: saline vehicle (Normal media), 14 ng L-T_4 /ml, or 14 ng L-T_4 /100 ml; in addition to BSA, 2 mg/ml: From a different set of cultures (without BSA), normal, D-T_4 (14 ng/ml) and L-T_4 (14 ng/ml) media were tested. All culture media had

maintained explanted duodenal quarters (either 4 or 8 quarters per dish) for 48 hr. The results of determinations of protein and alkaline phosphatase by the usual methods are shown in Table A-17.

After 48 hr of culture, protein levels were increased above control values in the media tested (Table A-17). Initially control media without BSA contained little protein, while those containing BSA showed higher protein contents (but not as great as the amount of protein which had been added). The increases in protein content after culture were larger in the BSA containing media than in media without BSA. Changes in protein content did not appear to correlate with the presence of hormone.

Alkaline phosphatase specific activity was variable; however, it appeared to increase in several cases (Table A-17). Phosphatase activity of the media did not show any consistent quantitative relationship to the presence of L-T_4. The largest specific activity was present in a culture containing L-T_4 (14 ng/ml), the pH of which had dropped below 7.2. Such cultures were ordinarily discarded, as the explants would be assumed to have been in poor condition. Therefore, both protein and phosphatase activity increased in the media following culture, and while the results were variable, the largest specific activities were found when L-T_4 was in the media. The number of explants cultured per dish could not be correlated to either protein or specific activity.

Table A-17 Protein Content and Alkaline Phosphatase Specific Activity of Culture Media

Media			Protein (mg/ml)	Alkaline Phosphatase Sp Act (μg/hr/mg protein/ml)
Controls (Uncultured)				
N			0.07	67.5
L(14/1)+BSA			1.25	16.5
Cultured (48 Hr)		Quarters/ Culture		
N+BSA	1	8	1.43	67.4
	2	4	1.61	75.5
	3	8	1.54	11.9
L(14/1)+ BSA	a	8	1.00	206.8
	b	4	1.57	124.6
	c	8	1.57	11.7
L(14/100) +BSA	I	8	1.16	124.9
	II	4	1.43	49.0
	III	8	1.45	17.7
N		4	0.07	22.6
D(14/1)		4	0.09	25.3
L(14/1)		4	0.09	34.8

Media were all from different culture dishes. The pH of culture 'a' was less than 7.2 (6.1). Specific activity was determined using protein content/ml.

END

03·03·80

FIN