A Comparison of the Elemental Concentrations in Tissue Thymocytes from Diabetic and Non-Diabetic Rats

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A COMPARISON OF THE ELEMENTAL CONCENTRATIONS IN TISSUE THYMOCYTES
FROM DIABETIC AND NON-DIABETIC RATS

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Abstract

The elemental concentrations in thymocytes from control and diabetic rats were studied by use of the techniques of cryofixation and X-ray microanalysis to determine whether any changes occur in the diseased state. Decreases in the concentrations of the elements P and K were found in thymocytes from the subcapsular and cortical regions of the gland in thymus tissue taken 4 days after the onset of diabetes. A decrease in the concentration of Mg was also found in thymocytes from the subcapsular region of the gland. These changes suggest that the metabolism of thymocytes in vivo is altered in the diabetic animals.

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Introduction

The thymus gland is the major site in the body in which thymocytes undergo development and differentiation. In this organ immature thymocytes undergo rapid cycles of division and as maturation proceeds the surface markers which are characteristic of mature lymphocytes are acquired (reviewed by Jordan and Robinson, 1981). Currently little is known about how division and maturation are controlled in vivo. However, studies on established cell lines in culture have shown that the inorganic cations K, Mg, Ca and particularly Na, are important in the control of cell division (see e.g., Rozengurt, 1980; Moolenaar et al. 1981; Frantz et al. 1981; Burns and Rozengurt, 1984). In many cell lines entry of Na into the cell, leading to an increase in intracellular pH, is thought to be a necessary signal for the initiation of DNA synthesis (Burns and Rozengurt, 1984; Moolenaar et al. 1984). The inorganic cations may play a role in the control of division in thymocytes. Alterations in levels of Na and K occur when isolated thymocytes are stimulated to divide by a mitogen (Averdunk, 1976; Felber and Brand, 1983; DeCoursey et al. 1984), and studies on tissue thymocytes using the techniques of cryofixation and X-ray microanalysis show that these changes may also occur in vivo (Warley, 1987a).

Atrophy of the thymus gland occurs in rats suffering from drug induced diabetes (Chatamra et al. 1983; Tabata et al. 1984; Chatamra et al. 1985). A similar weight loss from the thymus gland has also been described in the genetically diabetic BB Wistar rats (Chatamra et al. 1984). As yet the mechanism by which thymic atrophy is induced in the diabetic animals is not known, although the finding that atrophy of the thymus gland can be reversed by treating the diabetic animals with insulin (Tabata et al. 1984) suggests that the lack of this hormone in the circulation has a direct effect on the thymus gland. In order to determine whether alterations in elemental balance occur in thymocytes from diabetic animals thymic tissue was studied from drug induced diabetic and control animals, using the techniques of cryofixation and X-ray microanalysis.

Materials and Methods

Diabetes was induced in male CSE Wistar rats 250-300g body weight by a single injection of the drug streptozotocin (55mg / kg body weight i.p. made up in citrate buffer pH 4.0). Untreated male rats of the same weight range were used as control animals. Glucose levels in the urine were measured routinely using Klinistix to assess the development of diabetes, blood glucose levels were also determined. The operative procedures used were the same for both control and diabetic animals and these procedures have been outlined previously (Warley et al. 1987). The animals were killed under sodium pentabarbitone anaesthesia and the thymus gland was dissected out rapidly. Small pieces of tissue were fixed by plunging into stirred Freon 22 cooled in liquid nitrogen. The temperature of the coolant was measured using a Cu/Constantan thermocouple attached to a cooling rate meter (Emscope CRM 250). Under the conditions used for freezing cooling rates of $5000^{\circ}C/sec$ are achieved when measured on a bare thermocouple 75μm in diameter. Frozen material was stored under liquid nitrogen until sectioning.

Sections (approximately 300nm thick) were cut at $-65^{\circ}C$ to $-70^{\circ}C$ using a Slee cryoultramicrotome. Some of the later sections were cut on a LKB CryoNova, in order not to alter the experimental conditions a similar sectioning temperature was used. The sections were transferred onto Formvar or Pioloform coated grids and freeze dried for at least 1h in the nitrogen atmosphere of the cryostat. After drying the sections were placed in a Petri dish, surrounded by molecular sieve, and placed in a precooled desiccator in the cryostat. The sections were allowed to warm up to room temperature overnight, transferred to a carbon coater, still surrounded by molecular sieve, and coated with carbon before analysis. Analysis was carried out using an AEI EMMA 4 electron microscope fitted with a Link Analytical 860 series 2 EDS detection system. Spectra were collected over 100s live time, the area of the beam was adjusted to fit within the area of the section of a cell (usually about 4 μm diameter). The accelerating voltage was 60 kV and the beam current, measured with a Faraday cage, 4 nA. Spectra were processed and quantitative information was derived using the Quantem software supplied by Link Analytical. This program has facilities for gain calibration and for subtraction of contributions of grid and film from the measured continuum; the program is described in Gupta and Hall (1982). The system was calibrated by reference to standards made from known quantities of salts made up in 25% gelatin, full details of the standardisation are given in Kendall et al. (1985). Quantitative data were transferred directly to a mainframe computer using Declink software (Link Systems) and statistical analyses (one way analysis of variance and student's T-test) were carried out.

Results

The diabetic animals had high concentrations of

Table 1.
Concentrations of elements (mmol/kg dry wt. ± S.E.M.) in thymocytes from control rats.

Subcapsular Region.

Animal	n	Na	Mg	P	S	Cl	K
33	22	50±11	36±4	606±18	67±4	104±5	500±16
42	12	52±12	39±6	612±37	68±6	154±16	524±34
49	12	44±10	41±12	620±24	98±6	101±5	511±17
F		0.13	0.14	0.08	10.64 ***	10.40 ***	0.32

Cortical Region.

Animal	n	Na	Mg	P	S	Cl	K
33	29	40±8	30±5	626±40	87±6	160±9	522±27
42	23	38±8	28±6	706±50	103±26	126±17	606±39
49	22	47±8	23±6	643±26	108±4	91±9	551±26
50	13	31±12	26±5	598±18	84±9	126±7	521±14
F		0.41	0.34	1.18	0.62	6.29 ***	1.74

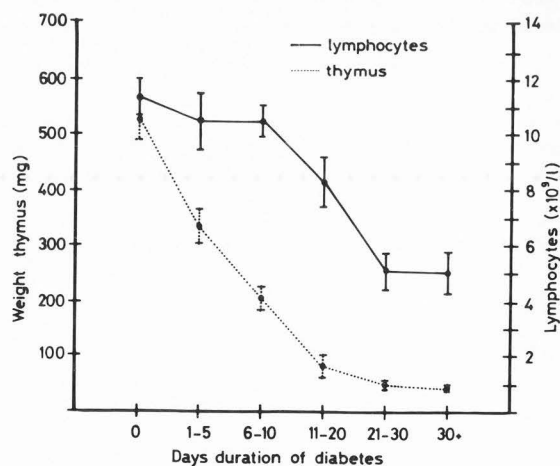*** $p < 0.01$, one way analysis of variance.

Fig. 1. Loss of weight from the thymus gland and decrease in numbers of lymphocytes in the circulation in male Wistar rats after the onset of diabetes. Results expressed as mean ± standard error.

glucose in their blood, the value for control animals was 4.46 ± 0.30 mmol/l (S.E.M. n= 23) at 4 days this had risen to 17.09 ± 0.63 mmol/l (S.E.M. n= 10), and to 23.03 ± 0.78 mmol/l (S.E.M. n= 10) by 20 days after the onset of diabetes. The weight of the thymus fell rapidly after the induction of diabetes, loss of weight from the thymus is shown in Fig. 1, these results resemble those previously published (Chatamra et al.
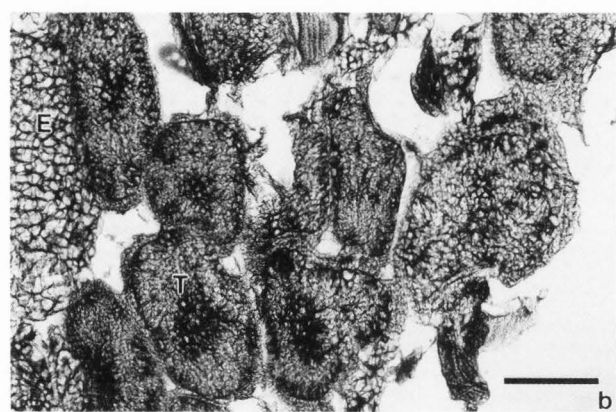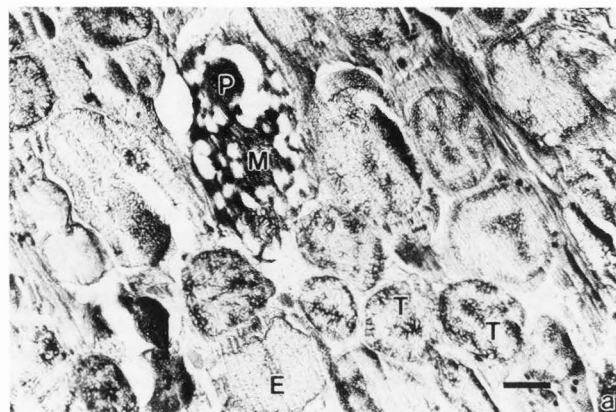
Fig. 2. Freeze dried frozen section from the cortical region of the thymus gland taken from a rat 4 days (a) and 16 days (b) after the onset of diabetes. In addition to the normal components of the thymus gland, thymocytes (T) and epithelial cells (E) a macrophage (M) containing a pyknotic inclusion (P) can be seen.

Marker = 2 μm

Table 2.

Concentrations of elements (mmol/kg dry wt. ± S.E.M.) in thymocytes from rats 4 days after onset of diabetes.

Subcapsular Region.

Animal	n	Na	Mg	P	S	Cl	K
31	21	19±7	21±5	501±12	104±6	83±4	410±10
47	23	42±10	29±4	567±25	83±6	125±10	472±19
55	5	40±36	24±12	529±53	85±2	111±30	510±55
F		1.5	0.69	2.59	3.36 *	6.02 **	5.25 **

Cortical Region.

Animal	n	Na	Mg	P	S	Cl	K
31	17	39±13	33±8	511±15	86±6	103±7	448±7
47	14	8±8	19±4	579±28	62±4	79±5	481±21
55	9	30±15	15±15	466±29	61±3	102±8	442±27
56	20	28±16	24±7	512±36	82±6	129±13	484±33
F		0.86	0.88	1.93	4.11 **	4.77 **	0.66

* $p < 0.05$, ** $p < 0.01$ one way analysis of variance.

animal 4 days after the onset of diabetes the presence of pyknotic cells within a macrophage can also be distinguished. The cells investigated in the present study were small thymocytes (T Fig.2). As the cytoplasm of the small thymocytes is sparse analyses were carried out with the electron beam covering the area of the section of a cell because of the difficulty in being sure that the probe could be confined within the cytoplasm.

Elemental composition of thymocytes from control animals

The elemental composition of thymocytes from the subcapsular and cortical regions of the thymus gland from the untreated control animals is shown in Table 1. The F statistic determined by one way analysis of variance and its level of significance are also shown in this table. The concentrations of the elements, apart from sulphur, fell within the range of values previously reported for these cells (Warley, 1987a).

The concentration of sulphur was generally lower than in previous studies. Concentrations of Fe, Ca and Zn were low, probably below the levels of detectability for this instrument. Concentrations of Na were lower and less variable than described previously (Warley, 1987a).

Analysis of variance showed that significant variation between the animals was found only for the elements S and Cl in thymocytes from the subcapsular region and in the element Cl in the cells from the cortical region of the gland. For elements other than S and Cl the animal to animal variation was within the range of the cell to cell variation.

When pooled cells from the subcapsular zone were compared with pooled results for thymocytes from the

1983; Chatamra et al. 1985). The number of lymphocytes in the circulation also decreased after the onset of diabetes, this fall in number began at about 10 days, by 20 days the number of circulating lymphocytes had decreased by a half (Fig. 1). Thymus glands from some of the animals in this group were taken for study by x-ray microanalysis.

The regions of the thymus gland used for study were the subcapsular and cortical regions. Identification of the regions was made on morphological grounds. The subcapsular region was identified by the presence of the connective tissue capsule, and subcapsular cells were defined as being within the first 5 cells underlying the capsule. Electron micrographs of freeze dried frozen sections from the cortical regions of the gland taken from animals 4 days and 16 days after the onset of diabetes are shown in Fig. 2a and b. The different cell types, the thymocytes and larger epithelial cells can be clearly seen. In the material taken from the

Table 3.
Concentrations of elements (mmol/kg dry weight ± S.E.M.) in thymocytes from rats 14+ days after the onset of diabetes.

Subcapsular Region.

Animal	n	Na	Mg	P	S	Cl	K
36	9	78±20	32±9	443±19	80±7	107±7	338±20
38	11	51±20	39±8	617±98	80±4	123±24	474±69
43	18	68±11	41±5	713±35	112±12	156±7	582±32
F		0.64	0.48	5.27*	3.43*	3.49*	7.43**

Cortical Region.

Animal	n	Na	Mg	P	S	Cl	K
36	27	49±7	34±5	748±17	99±5	124±5	610±13
38	18	40±19	27±7	648±38	100±9	109±7	560±35
43	12	65±9	42±9	842±74	122±9	190±14	705±68
F		0.75	1.09	5.47**	2.43	25.63***	3.88*

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$
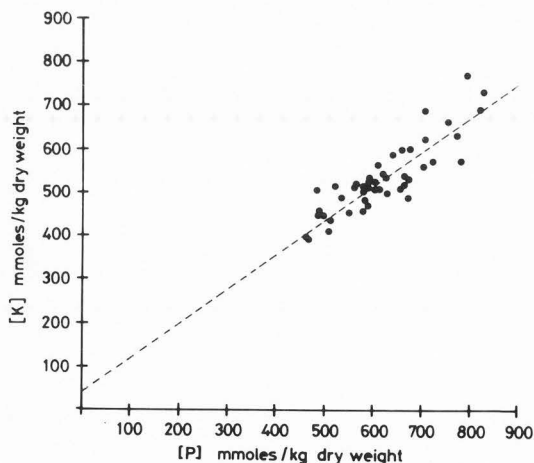one way analysis of variance.

Fig. 3. Correlation between the elements K and P in thymocytes from the subcapsular region of control animals

cortical region significant increases in the elements S and K were found ($p < 0.05$ Student's t-test) in the thymocytes from the cortical region.

Elemental concentrations in thymocytes from animals 4 days after the onset of diabetes

Concentrations of elements in thymocytes from the

Table 4.
Comparison of results for the control animals with those from the diabetic animals.

Subcapsular Region.

Time diabetic	n	Na	Mg	P	K
0	46	49±6	38±4	611±14	509±12
4	49	32±7	25±3*	535±14**	449±12***
14+	38	66±9	38±4	621±37	493±30

Cortical Region.

Time diabetic	n	Na	Mg	P	K
0	87	40±4	27±3	647±20	552±15
4	60	26±7	24±4	520±15***	467±13***
14+	57	50±7	33±4	736±23**	614±20*

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$
Student's t-test

subcapsular and cortical regions of the thymus gland from animals 4 days after the onset of diabetes are shown in Table 2. The F statistic and levels of significance of results for the analysis of variance between the individual animals in the group are also shown in this Table. In the diabetic animals the concentration of K was more variable in thymocytes from the subcapsular region than in the control animals. Analysis of variance showed that there was significant variation in this element between the different animals. The increase in concentration of K found in thymocytes from the cortical region when compared with cells from the subcapsular zone seen in the control animals (Table 1 and Warley, 1987a) was not observed in thymocytes from these diabetic animals.

Elemental concentrations in thymocytes from animals 14+ days after the onset of diabetes

Thymocytes were analysed in tissue taken from animals which had been diabetic for 14, 16 and 17 days, these animals were grouped together. Concentrations of elements in thymocytes from these animals are shown in Table 3. There was more variation in the concentrations of elements between the different animals in this group compared to the control group of animals. In addition to the variation in S and Cl significant variation between animals was found in the elements P and K in thymocytes from both the subcapsular and cortical regions.

Comparison of results from diabetic animals with those of the control animals

For each group of animals the results for each region were pooled separately so that comparisons could be made between the diabetic and control animals. As

previous studies (Warley, 1986) had shown that the concentrations of S and Cl can vary between individual animals these elements were omitted. Comparison of results from the animals 4 days after the onset of diabetes (Table 4) with results from the control animals showed that in the diabetic animals thymocytes from the subcapsular region showed statistically significant decreases in the concentrations of Mg, P and K. Significant decreases in P and K also occurred in the cells from the cortical zone. Fewer differences were seen in thymocytes from animals 14+ days after the onset of diabetes, significant increases were found in P and K in the cells from the cortical region. However, in this group of animals pooling of the results does mask the considerable variation that is seen between the individual animals.

Correlation between the elements K and P

In control animals a strong correlation was found between the elements K and P in the thymocytes from both the subcapsular (r= 0.85; p < 0.001) and cortical (r= 0.90; p < 0.001) regions. The relationship between K and P in thymocytes from the subcapsular region of the control animals is shown in Fig. 3. Strong correlation between P and other elements was not found. This correlation between the elements P and K was also found in the thymocytes from the diabetic animals. At 4 days after the onset of diabetes the correlation coefficient between K and P for the subcapsular region was 0.84 (p <0.001) and for the cortical region 0.88 (p <0.001) and at 17 days 0.96 (p <0.001) and 0.9 (p <0.001). The strong correlation between these elements is also reflected in the values for the ratio of K/P. In the cortical region for normal animals the value was 0.96 ± 0.035; in the 4 day diabetics 0.9 ± 0.012 and at 17 days 0.839 ± 0.012. There is no significant difference between any of these values.

Discussion

When any tissue is studied by X-ray microanalysis variation in the results can be introduced by problems either in the quantitative or specimen preparation procedures. Generally when continuum normalisation is used for quantitative purposes the results obtained are affected by any factors which lead to inaccuracies in the estimation of the continuum (Hall, 1979; Gupta and Hall, 1982; Hall and Gupta, 1983). The extent to which analytical and preparative procedures may affect results obtained in our system has been discussed previously (Warley, 1987a) and the conditions used in the present study should not lead to artefactual estimations of concentrations. In addition, in the present study thymocytes were analysed using a wide probe to cover the whole area of the cell, because a larger volume is analysed this should lead to less variation in the results. In the control animals analysis of variance showed that significant differences between the animals were seen only in the elements S and Cl. Previous studies (Warley, 1986) have shown that the concentrations of these elements tend to vary between individual animals. Quantitation of S can be affected

by loss of this element from the specimen when analysis does not take place on a cold stage (Rick et al. 1979) and Cl may selectively contaminate some structures (Hall and Gupta, 1982). As the specimens were quench frozen in Freon this could act as a source of Cl contamination. However, if non-specific contamination with Cl was occurring more variability and higher concentrations of Cl might be expected. At present non-specific sources for the variability in concentrations of S and Cl cannot be ruled out. In view of the variation in concentration of S and Cl that was seen in the control animals these elements were not used for comparison between the control and diabetic animals.

One of the interesting features to emerge from the present study is the strong correlation found between the elements K and P in the thymocytes. Thymocytes consist mainly of nucleus which contains dense heterochromatin. The analyses reported in this paper therefore reflect nuclear concentrations of elements. Other studies using X-ray microanalysis, particularly those on nucleated erythrocytes which also contain high levels of heterochromatin, show that high levels of K and P are found in the nuclei (Jones et al. 1979; Andrews et al. 1983). Investigations on psoriatic skin have also shown high levels of K and P in the nuclei (Grundin et al. 1985), these authors ascribed the finding to increased levels of nucleic acids in the dividing cells. More recently von Zglinicki and Bimmler (1987) have described higher concentrations of K associated with the heterochromatin in nuclei of liver cells. Biochemical studies on chromatin in isolated nuclei indicate that chromatin remains condensed in media of high ionic strength (Olins and Olins, 1972; and see discussion in Jones et al. 1979). The close relationship found between K and P in thymocytes suggests that K may be associated with condensed chromatin in vivo.

In the present study the elemental composition of thymocytes was examined in tissue taken from diabetic animals 4 days after the onset of diabetes and also at a later stage 14+ days after the onset of diabetes. These time points represent two different stages in atrophy of the thymus gland. At 4 days the thymus gland is undergoing rapid loss of weight, any changes in elemental concentration at this time may be related to this process. At 14+ days the loss of weight from the gland is almost complete; the cells remaining in the gland at this time probably reflect a residual population or populations not sensitive to insulin. Bearing this in mind, it is interesting that decreases in elemental concentrations are found in the cells examined from animals 4 days after the onset of diabetes. The following reasons suggest that the decreases are due to the disease process rather than problems inherent in microanalysis. Firstly, using the same conditions of analysis little variation was observed in the concentrations of these elements in thymocytes from the control animals. Secondly, there is a significant difference between the mean values for these elements between the control and 4 day diabetic animals and within each group of animals there is

little variation in the spread of values for the individual cells. Thirdly, in the animals taken 4 days after the onset of diabetes there is no increase in K concentration in the cells from the cortical region of the thymus compared to the subcapsular region.

The differences noted between the control and diabetic animals at 4 days after the onset of diabetes are not excessive, but only slight changes may be necessary to cause alteration in the metabolism of the cell. There is variability between the different animals but this might be expected in view of the heterogeneity of the thymocyte population. Although increased uptake of K into tissues after the administration of insulin is a well recognised phenomenon, little is known about the concentration of this element in tissues from long term diabetic individuals. The majority of studies on the effects of diabetes on the levels of elements in tissues are on skeletal muscle (Moore, 1983). In the present paper loss of K from thymocytes in vivo is recorded. For both the subcapsular region and the cortical region a decrease in K of 11% was noted this decrease could alter the metabolism of the cell as the concentration of K can affect rates of protein synthesis (Lubin, 1982). A significant decrease in the concentration of Mg occurs in thymocytes from the subcapsular region of the gland, this might be expected to affect the basic metabolism of the cell as Mg is required as a cofactor for the activity of many enzymes. A similar change (34% decrease) to that of Mg is also seen for the element Na in thymocytes from the subcapsular region. This change is not significant as the larger standard error on the measurement makes the observation statistically less reliable. The decreases recorded in this paper are measured in thymocytes which retain normal morphology, and may presage the greater loss of elements found in those cells which have undergone pyknotic degradation (Warley, 1987b).

Although it has been shown that in mature thymus derived lymphocytes insulin receptors are found only on activated cells (Krug et al. 1972; Helderman and Strom, 1977; Helderman and Strom, 1978)) little has been described about insulin receptors and insulin sensitivity of thymocytes within the gland. Insulin receptors have been shown to be present on thymocytes isolated from rodents (Goldfine et al. 1972; Soll et al. 1974; Okuno et al. 1983). Interestingly increased binding of insulin has been described in large and medium size thymocytes (Okuno et al. 1983) which are the cells about to undergo division. Thus for immature thymocytes sensitivity to insulin may also be indicative of actively dividing cells. Insulin stimulates the uptake of K into many tissues (Moore, 1983), this enzyme stimulates Na/K ATPase in lymphocytes (Hadden et al. 1972) and in epithelial cell insulin also stimulates the Na/H exchange mechanism (Vara and Rozengurt, 1985). The decrease in K in thymocytes of the 4 day diabetic animals could be due to cessation of stimulation due to withdrawal of insulin.

The results presented in this paper are interesting not only because they give some insight into the sensitivity of thymocytes to insulin but also because they yield information about the maturation pathway of thymocytes. It has been shown that diabetes induced atrophy of the thymus gland is due to a loss of cells from the cortical region of the gland (Tabata et al. 1984; Warley et al. 1987). The role of cortical thymocytes as precursors of circulating lymphocytes has been questioned (for review see Scollay, 1983). The results presented here agree with the view (Osmond, 1985; Rocha, 1985) that the cortical thymocytes are a source of short lived lymphocytes entering the circulation.

Acknowledgements

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Discussion with Reviewers

T. von Zglinicki: Is the variation within every experimental group affected by the inclusion of different amounts of cytoplasm into the measuring area?

Author: The cytoplasm of the small lymphocyte is sparse and represents a very low fraction of the dry weight of the cell so I feel that the contribution of this compartment to the overall analysis will be negligible.

T. von Zglinicki: A similar decrease between control and 4 days group of all the elements measured is not what would be expected in the case of major changes of functional state of cells or nuclei. Could there be any changes in the overall degree of hydration as seen by the compactness of the cells with respect to nuclei?

G. M. Roomans: In the present experimental situation expressing the data in terms of mmol/kg dry weight could be misleading, the decrease in concentration af-

ter 4 days and subsequent general increase might be due to a change in organic matrix of the cell.

Author: I agree that changes seen after the induction of diabetes could reflect alterations occurring in the cell water content. I have estimated the water content of isolated thymocytes from 3 animals 4 days after the onset of diabetes. These thymocytes were isolated in autologous serum in order to maintain the tonicity of the medium in the in vivo state. The water content was 77.6 ± 1.9% (SD n=3) which compares with an earlier estimation of water content of control thymocytes of 73.6 ± 4.4% (SD n=3). Because of the spread of values for the control cells it is difficult to tell whether there is a significant increase in cell water content in the cells from diabetic animals.

G. M. Roomans: Could you explain how your data support the theory that cortical thymocytes are a source of short lived lymphocytes?

P.O. Berggren: How do the results from the present study agree with the view that the cortical lymphocytes are a source of short lived lymphocytes entering the circulation? Of what origin are the lymphocytes that decrease? Why did the number of lymphocytes not decrease until after 20 days and why did they never decrease to less than about 50% of the control number?

Author: The results presented here show that there is a delay after the thymus gland atrophies before there is a decrease in the number of lymphocytes in the circulation. It is thought that mature thymocytes do not pass directly into the circulation, but may reside for sometime in the spleen. The delay seen in the decrease of lymphocytes in the circulation probably reflects the time taken for cell which have matured in the thymus before the onset of diabetes to reach the circulation. The thymus acts as a source of T cells for the circulation. B lymphocytes usually only comprise about 20% of the circulating white cells in the normal animal. The fact that total lymphocyte numbers do not decline below 50% of the values for the control animals suggests that some T lymphocytes remain in the circulation even after almost complete atrophy of the thymus gland. Our preliminary studies using monoclonal antibodies to rat T and B cells have indeed indicated that the decrease is due to a decrease in the number of T cells in the circulation. Thus it appears that the decrease of 50% in circulating lymphocytes is due to removal of a short lived population of lymphocytes which is not replaced by cells from the atrophied gland.