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Effect of hypothyroidism on the lipid composition and fluidity of rat colonic apical plasma membranes

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Prior studies have suggested that the lipid composition and lipid fluidity of cellular membranes of various organs are altered in the hypothyroid rat. To date, the effects of hypothyroidism on these parameters have not been examined in rat colonic apical plasma membranes. In the present experiments, male Sprague-Dawley rats were fed a pelleted diet (control group) or the same diet containing 0.1% propylthiouracil (hypothyroid group) for 3 weeks. The lipid composition and lipid fluidity of apical plasma membranes prepared from colonocytes of these two groups of animals were then examined and compared. Membranes prepared from the hypothyroid animals were found to possess a higher level of linoleic acid (18:2) and a lower level of arachidonic acid (20:4) than membranes from control animals. The molar ratio of cholesterol/phospholipid was also lower in hypothyroid membranes secondary to a decreased cholesterol content compared to their control counterparts. Moreover, the lipid fluidity of colonic apical plasma membranes, as assessed by (1) the ratio of excimer to monomer fluorescence intensities of the lipid-soluble fluorophore pyrenedecanoic acid and (2) the anisotropy values of the fluorophore DL-12-(9-anthroyloxy)stearic acid using steady-state fluorescence polarization techniques, was greater in hypothyroid animals. These data, therefore, indicate that alterations in the lipid composition and fluidity of colonic apical plasma membranes can be detected in hypothyroid rats.

Introduction

Considerable evidence now exists that a number of structural and functional alterations are present in various organs, particularly the liver, of animals with experimentally induced hypothyroidism [1–10]. For example, the levels of unsaturated fatty acids in the phospholipids of hepatic mitochondrial, microsomal and nuclear membranes of hypothyroid rats have been shown to be abnormal [1–4]. Additionally, the lipid composition of erythrocyte membranes [10], serum lipid levels [11], as well as the activation of several hepatic intracellular membrane-bound enzymes [3,6,8,12] have been found to be altered in hypothyroid animals. In an attempt to explain these diverse membrane changes, previous investigators [3,4,12,13] have suggested that the membrane lipid alterations noted in hypothyroid animals might

Abbreviations: 12-AS, DL-12-(9-anthroyloxy)stearic acid; POPOP, 1,4-bis(2-(5-phenyloxazolyl))benzene; T_3 and T_4, tri- and tetraiodothyronine.

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lead to changes in their 'lipid fluidity' * which, in turn, would affect various membrane functions. It should be noted, however, that most of these previous studies failed to measure directly the lipid fluidity of these membranes [3,12,13]; rather, changes in fluidity were inferred, based on alterations in membrane lipid composition. Additionally, relatively few studies, to date, have examined the effects of hypothyroidism on cell surface membranes [10].

In this regard, during the past several years, workers in our laboratory have examined and described studies of the lipid dynamics and lipid–protein interactions in the antipodal plasma membranes of rat colonic epithelial cells [14–20]. These studies have demonstrated that: (1) a number of cell surface functions, including certain transmembrane transport processes and enzymatic activities, appear to be modulated by alterations in the lipid fluidity of these membranes [14–20]; and (2) the physical state of the lipids of colonic plasma membranes is altered in various experimental models of disease [18,20].

These latter observations, together with the findings of previous studies (see above) as well as the recognized alterations in large intestinal function which accompany hypothyroidism [21,22], have led us to explore the possible effects of propylthiouracil-induced hypothyroidism on the lipid composition and fluidity of rat colonic apical plasma membranes. The results of these studies, along with a discussion of their possible physiological significance, serve as the basis for the present report.

Methods and Materials

Animals and dietary protocol

Male Sprague-Dawley rats weighing 150–175 g were maintained either on a pelleted diet (Camm Maintenance Rodent Diet) or on the same diet containing 0.1% propylthiouracil, for 3 weeks. As previously described [23], the propylthiouracil was dissolved in hot ethanol, added to the pelleted diet, and vigorously mixed. Ethanol alone was added to the control diets. The ethanol was allowed to evaporate off under a fume hood for 24 h. Diets were prepared weekly and stored in sealed containers at 4 °C.

After 3 weeks, the group of animals consuming propylthiouracil showed a tendency for less weight gain than their respective controls (e.g., final weights, propylthiouracil-containing diet, 230 ± 17 g ($N = 24$) versus control, 250 ± 20 g ($N = 24$)); this difference, however, was not found to be statistically significant. Recent studies by our laboratory [23] had established that rats administered 0.1% propylthiouracil for 3 weeks in their diets possessed significantly lower serum T_4 and T_3 levels than their control counterparts.

Preparation of colonic apical membranes

After three weeks on their respective diets, animals from both groups were fasted overnight with water ad libitum before being killed. The colons were excised, the cecum discarded and epithelial cells, relatively devoid of goblet cells, were obtained using a technique which combined chelation of divalent cations with mild mechanical dissociation, as described [15].

Apical plasma membranes from these cells were then prepared as described by Brasitus and Keresztes [15]. The purity of the membrane preparations and the degree of contamination by intracellular membranes were evaluated using appropriate marker enzymes [15]. The specific activity ratio ((purified apical membranes)/(crude homogenate)) for the apical enzyme marker cysteine-sensitive alkaline phosphatase (*p*-nitrophenylphosphatase) [15] was approx. 13–16 in all membrane preparations. The corresponding specific activity ratios for NADPH–cytochrome-*c* reductase, succinic dehydrogenase and Na^+/K^+-dependent ATPase, marker enzymes for microsomal, mitochondrial and basolateral membranes, respectively, ranged from 0.40 to 1.30 in all preparations. Membranes were suspended in the appropriate buffer (see below) and used immediately.

Compositional studies

Lipids were extracted from the apical membranes by the method of Folch et al. [24]. Phos-

* The term 'lipid fluidity' as applied to natural membranes is used in this paper to denote the relative motional freedom of the lipid molecules or substituents thereof. A more detailed description has been previously published [32].

pholipids [25] and cholesterol [26] were measured as described previously. The neutral lipid and phospholipid compositions of the extracts were further analyzed by thin-layer chromatography according to the method of Katz et al. [27]. Fatty acids of the total lipid extracts were derivatized and the fatty acid methyl esters were analyzed on a Hewlett-Packard 5790A gas-liquid chromatograph equipped with a flame ionization detector as described previously [28]. Protein was estimated by the method of Lowry et al. [29].

Liposomes

Liposomes were prepared from extracted membrane lipids as described previously [30]. Briefly, lipid was extracted from the membranes as described by Folch et al. [24], and approx. 0.3–0.5 mg of dried lipid was suspended in phosphate-buffered saline [30]. The suspension was then sonicated under N_2 at 4°C for 5–10 min. Thereafter, the preparations were centrifuged at 10000 $\times g$ for 10 min and the resulting supernatant was used for fluorescence studies (see below).

In certain studies, liposomes were prepared as described above using cholesterol and synthetic phosphatidylcholine (L-β-oleoyl-γ-palmitoyl-2-phosphatidylcholine) to obtain preparations with cholesterol/phospholipid molar ratios of 0.80 and 0.67 for fluorescence studies (see below).

Fluorescence studies

Excimer and monomer fluorescence intensity studies. Excitation and emission spectra and estimation of total fluorescence intensity were obtained with a Perkin-Elmer 650-40spectrofluorometer equipped with a multitemperature cuvette holder, using the lipid-soluble fluorophore pyrenedecanoic acid (Molecular Probes, Junction City, OR) at 25°C. Samples and buffer were thoroughly deoxygenated and saturated with N_2 prior to examination, and a nitrogen atmosphere was maintained within the sample chamber [31]. The samples were loaded with pyrenedecanoate as described previously [32]. The content of the fluorophore in each preparation was estimated fluorometrically as described by Cogan and Schachter [32]. Final molar ratios of probe/lipid ranged from 0.001 to 0.003 and the excimer/monomer differences noted in the samples (see below) could not be ascribed to differences in the probe concentration in the preparations.

Excimer and monomer fluorescence intensities were determined at an excitation wavelength of 345 nm and emission wavelengths of 397 nm (monomer) and 465 nm (excimer). Both excitation and emission slits were set at 4 nm. Measured intensities were corrected as necessary for background fluorescence of the buffer and/or unlabeled samples [33]. Each measurement was repeated at least three times. For a given sample, the precision in the ratio of the excimer to monomer fluorescence intensities was better than $\pm 3\%$.

To examine the effects of temperature on the excimer/monomer ratio of pyrenedecanoate in control and hypothyroid membranes, this ratio was determined in these preparations over the range of 10–40°C. The logarithm of this ratio was plotted against $1/T$ (K^{-1}) to detect 'lipid thermotropic transitions' * as described [14,15].

Excimer and monomer lifetimes of the excited state for pyrenedecanoate in each preparation, using phase and modulation values determined at 6 MHz modulation frequency relative to a POPOP reference solution [34], were determined at 25°C as described by Hresko et al. [33] with an SLM 4800 spectrofluorometer (SLM-Aminco, Urbana, IL). No significant differences in the excited-state lifetimes of the probe in the various preparations were noted in the present studies (not shown).

Steady-state fluorescence polarization studies. Steady-state fluorescence polarization studies were also performed with a Perkin-Elmer 650-40 spectrofluorometer adapted for fluorescence polarization using the lipid soluble fluorophore DL-12-(9-anthroyloxy)stearic acid (12-AS) (Molecular Probes). The methods used to load the membranes and quantification of fluorescence have been discussed extensively in publications from this laboratory [13,14,18]. Final molar ratios of probe/lipid ranged from 0.001 to 0.002 and the anisotropy (r) differences noted in these studies could not be ascribed to differences in the probe con-

* The term 'lipid thermotropic transition' is used in a general sense to denote a thermally induced alteration in the physical state of the lipid. This change might involve lipid clusters, order–disorder transitions of the liquid-crystalline-to-gel type, lateral phase separations, or other mechanisms [37,53].

centrations in these preparations. Corrections for light scattering (membrane suspensions minus probe) and for fluorescence in the ambient medium (quantified by pelleting the membranes after each estimation) were made routinely and the combined corrections were less than 4% of the total fluorescence intensity observed for 12-AS-loaded suspensions. The lifetime of 12-AS in each preparation was estimated by phase fluorometry at 30 MHz in an SLM 4800 subnanosecond polarization spectrophotometer as described previously by workers in this laboratory [35]. No significant differences in the excited-state lifetimes of the probe in the various preparations were noted in the present experiments (not shown).

Statistical methods

All results are expressed as mean values ± S.E. Paired or unpaired *t*-tests were used for all statistical analysis. $P < 0.05$ was considered significant.

Materials

Unless otherwise indicated, all materials were obtained from Fisher Chemical Co. (Fair Lawn, NJ) or Sigma, (St. Louis, MO).

Results

Lipid composition

The effects of propylthiouracil-induced hypothyroidism on the neutral lipid and phospholipid composition of rat colonic apical plasma membranes are shown in Table I. As can be seen from this table, the content and percentage of cholesterol in plasma membranes prepared from rats administered propylthiouracil were significantly decreased as compared to the values obtained in their control counterparts. In contrast, no significant differences were noted in the other neutral lipids, phospholipid content and individual phospholipids of control and hypothyroid membranes.

Compositional analysis of the total fatty acids of control and hypothyroid membranes, furthermore, revealed that the levels of linoleic acid (18 : 2) and arachidonic acid (20 : 4) were significantly higher and lower, respectively, in the colonic plasma membranes prepared from the propylthiouracil-treated animals (Table II).

Prior studies in biological membranes and model bilayers have shown that differences in the ratios of cholesterol/phospholipid (mol/mol), sphingomyelin/phosphatidylcholine (mol/mol), and protein/lipid (wt./wt.) as well as in the degree of saturation of fatty acids of phospholipids were associated with changes in the physical state of membrane lipids [36–41]. As shown in Table I, no differences were noted in the ratios of sphingomyelin/phosphatidylcholine (mol/mol) and protein/lipid (wt./wt.) or in the saturation index of control and hypothyroid membranes. In contrast to these findings, however, hypothyroid membranes were found to possess a significantly lower molar ratio of cholesterol/phospholipid than their control counterparts (Table I). Differences in the levels of cholesterol rather than in the levels of phospholipid in these membranes

TABLE I

COMPOSITIONAL ANALYSIS OF LIPID EXTRACTS OF COLONIC APICAL MEMBRANES IN CONTROL AND HYPOTHYROID RATS

Values are expressed as percent (wt/wt) and represent means ± S.E. of eight determinations of four separate preparations of each membrane. * $P < 0.05$ or less compared to control values.

Component	Composition (%, w/w)	
	control	hypothyroid
Cholesterol	25.47 ± 1.42	21.63 ± 1.06 *
Cholesterol esters	1.10 ± 0.31	1.16 ± 0.24
Fatty acids	8.93 ± 0.79	9.20 ± 0.86
Triacylglycerols	1.62 ± 0.45	1.59 ± 0.39
Phosphatidylethanolamine	10.77 ± 1.73	11.25 ± 1.02
Phosphatidylinositol	7.14 ± 0.87	7.92 ± 1.59
Phosphatidylserine	4.36 ± 0.80	4.48 ± 1.18
Phosphatidylcholine	26.95 ± 2.44	26.44 ± 2.29
Sphingomyelin	14.80 ± 0.39	14.07 ± 0.65
Lysophosphatidylcholine	1.57 ± 0.27	1.72 ± 0.46
Cholesterol/phospholipid (mol/mol)	0.80 ± 0.03	0.67 ± 0.03 *
Sphingomyelin/lecithin (mol/mol)	0.55 ± 0.02	0.53 ± 0.02
Protein/lipid (wt/wt)	0.63 ± 0.03	0.64 ± 0.04
Saturation index [a]	0.47 ± 0.03	0.48 ± 0.01
Cholesterol/protein (w/w)	0.12 ± 0.01	0.09 ± 0.01 *
Phospholipid/protein (w/w)	0.29 ± 0.03	0.27 ± 0.02

[a] Calculated as (the total number of saturated residues)/(the sum of the number of each type of unsaturated residue multiplied by the number of double bonds in that residue).

TABLE II

COMPOSITIONAL ANALYSIS OF TOTAL FATTY ACIDS OF COLONIC APICAL MEMBRANES OF CONTROL AND HYPOTHYROID RATS

Values represent means ± S.E. of eight determinations of four separate preparations of each membrane. * $P < 0.05$ compared to control values.

Fatty acid	Composition (%, w/w)	
	control	hypothyroid
16:0	28.0 ± 0.81	27.9 ± 0.55
16:1	2.5 ± 0.23	2.3 ± 0.24
18:0	19.2 ± 1.45	17.7 ± 0.32
18:1	24.0 ± 0.81	24.8 ± 0.46
18:2	15.2 ± 0.86	18.8 ± 0.46 *
20:4	11.2 ± 1.06	8.5 ± 0.74 *

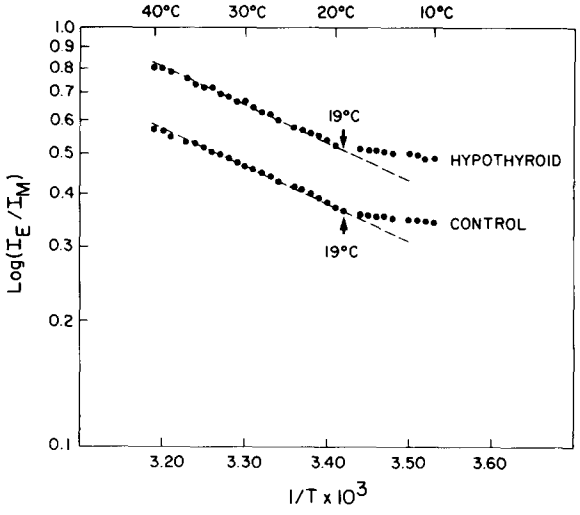

Fig. 1. Representative Arrhenius plots of the excimer/monomer ratio of pyrenedecanoate in rat colonic apical plasma membranes prepared from control and hypothyroid animals.

appeared to account for this latter finding (Table I).

Fluorescence studies

In view of the difference in the molar ratios of cholesterol/phospholipid in these colonic plasma membranes, it was of interest to examine and compare their lipid fluidity, using the ratio of excimer to monomer fluorescence intensities of pyrenedecanoate [32] (Table III). As shown in this table, hypothyroid membranes and liposomes were both found to possess significantly higher excimer/monomer intensity ratios than their control counterparts, an indication of greater lipid fluidity [32]. In this regard, it should also be noted that each of the liposome preparations possessed higher excimer/monomer ratios than their respective intact membranes, suggesting that proteins in each membrane appeared to influence the lipid fluidity of these membranes as assessed by this technique.

The effects of temperature on the excimer/monomer ratio of pyrenedecanoate in control and hypothyroid colonic apical membranes are illustrated by representative Arrhenius plots in Fig. 1. The plots appear reasonably linear from 40 °C to approx. 19–20 °C, but a distinct change in slope then occurs with further cooling. Transition temperatures for control (19.4 ± 1.2 °C, $N = 3$) and hypothyroid membranes (19.0 ± 0.8 °C, $N = 3$) were found to be similar. As can be seen in Fig. 1, however, at every temperature examined, hypothyroid membranes were found to possess a higher excimer/monomer ratio than control membranes.

TABLE III

FLUORESCENCE STUDIES USING PYRENEDECANOATE AND 12-AS OF RAT COLONIC CONTROL AND HYPOTHYROID APICAL MEMBRANES AND THEIR LIPOSOMES

Values represent means ± S.E. N, number of preparations. * $P < 0.05$ or less compound to control value of each type of preparation. ** $P < 0.05$ or less compound to respective membrane values.

Preparation	N	Excimer/monomer ratios of pyrenedecanoate	N	Mean fluorescence anisotropy, r, of 12-AS
Control membranes	4	0.42 ± 0.03	3	0.108 ± 0.003
Control liposomes	4	0.58 ± 0.03 **		–
Hypothyroid membranes	4	0.58 ± 0.06 *	3	0.092 ± 0.002 *
Hypothyroid liposomes	4	0.77 ± 0.03 *,**		–

Since pyrenedecanoate, due to its pyrene moiety, tends to partition into more fluid domains, such changes in probe distribution may affect its local concentration and the excimer to monomer ratio [33,42]. It was, therefore, important to use another fluorophore such as 12-AS, which does not have any differential partition in the membrane plane [43], to examine the fluidity of control and hypothyroid membranes. As shown in Table III, the anisotropy, (r) values of 12-AS, which vary inversely with fluidity [43], were higher in control membranes, again indicating that hypothyroid membranes were more fluid than their control counterparts.

Finally, since the differences in the fluidity of control and hypothyroid membranes appeared to be due, at least in part, to changes in their cholesterol/phospholipid molar ratios, it was of interest to determine whether these rather small changes (Table I) were indeed responsible for the membrane's fluidity differences. To address this issue, modeling studies were performed using cholesterol/phospholipid liposomes with molar ratios of 0.80 and 0.67, in order to mimic the cholesterol/phospholipid ratios of control and hypothyroid membranes, respectively. Analysis of the excimer/monomer ratio of pyrenedecanoate in these preparations revealed values of 0.37 ± 0.03 and 0.24 ± 0.03 ($N = 3$, $P < 0.01$) for the model liposomes with cholesterol/phospholipid molar ratios of 0.67 and 0.80, respectively. It would, therefore, appear that changes in the cholesterol content and cholesterol/phospholipid molar ratio could explain the fluidity differences between control and hypothyroid membranes.

Discussion

The present studies demonstrate for the first time, to our knowledge, that the lipid composition and lipid fluidity of colonic apical plasma membranes are altered in hypothyroid rats. The latter animals were found to possess higher levels of linoleic acid and lower levels of arachidonic acid in their colonic plasma membranes than control animals. In agreement with these findings, prior investigations in hypothyroid rats have revealed similar alterations in the levels of these two fatty acids in hepatic mitochondrial, microsomal and nuclear membranes [1–3,12].

The mechanism(s) responsible for the changes in colonic membrane fatty acids levels in hypothyroid rat noted in the present studies are unclear at this time. In this regard, however, prior investigations in the liver of hypothyroid animals have suggested that alterations in the activities of various enzymes, including those involved in fatty acid desaturation [3,12] and fatty acid elongation [6,12] as well as phospholipase A_2 [5], fatty acid synthetase [12] and acetyl-CoA carboxylase [12] might be responsible for the abnormal levels of linoleic and arachidonic acids seen in the hepatic intracellular membranes of these animals. Further studies along these lines will, therefore, be necessary to clarify this issue.

In the present studies, the content of cholesterol was also found to be decreased in colonic apical plasma membranes prepared from hypothyroid rats. Since the phospholipid content was unchanged, this resulted in a lower cholesterol/phospholipid molar ratio in these membranes compared to their control counterparts. In contrast to these findings, earlier studies in hypothyroid rats failed to detect changes in the cholesterol content of hepatic intracellular membranes [1–3,12]. Ruggiero et al. [10], however, have reported a decrease in the cholesterol content of rat erythrocyte membranes in hypothyroidism. In the latter investigation, however, the phospholipid content of erythrocyte membranes was also decreased, so that the molar ratio of cholesterol/phospholipid was not altered in these membranes [10]. The mechanism(s) responsible for producing the decreased cholesterol content in rat colonic hypothyroid membranes in the present studies is, therefore, also unclear at this time.

In this regard, however, previous studies in this laboratory have demonstrated a relationship between cholesterol absorption, endogenous cholesterol synthesis and plasma membrane cholesterol content in the intestinal track [44], i.e., increased absorption led to decreased synthesis which, in turn, resulted in a lower apical membrane cholesterol content. Although endogenous large intestinal cholesterol synthesis was not examined in the present experiments, we have shown recently that cholesterol absorption is increased in the intestinal tract of propylthiouracil-induced hypothyroid rats [23]. While speculative, it would

seem reasonable to suggest that reduced endogenous cholesterol synthesis, secondary to increased absorption of this sterol in hypothyroid rats, might be responsible for the decreased cholesterol content and lower cholesterol/phospholipid molar ratio seen in the colonic apical plasma membranes of these animals. Further studies, however, will be necessary to clarify this issue.

Regardless of the mechanism(s) involved, however, it does appear that colonic apical plasma membranes of hypothyroid rats do have a decreased ratio of cholesterol/phospholipid (mol/mol) compared to membranes prepared from control animals. As noted earlier, a decrease in this parameter would be expected to increase the lipid fluidity of rat colonic apical membranes [38,39]. In the present studies, the lipid fluidity of these membranes, as assessed by the ratio of excimer/monomer fluorescence intensities of pyrenedecanoate as well as by the anisotropy values of 12-AS, was, indeed, higher than in their control counterparts. Similar findings were also noted in studies performed on the lipid extracts (liposomes) of these membranes using pyrenedecanoate. It would, therefore, appear that the decreased molar ratio of cholesterol/phospholipid in the hypothyroid membranes was, at least in part, responsible for their greater fluidity. The results of the liposome modeling experiments with pyrenedecanoate lend additional support to this contention. No differences were detected in several other compositional parameters which have been shown previously to influence the fluidity of model bilayers and natural membranes (Table I).

It should be noted that the lipid fluidity of control and hypothyroid membranes was assessed with the lipid-soluble fluorophore pyrenedecanoate in these studies. Pyrene probes, such as pyrenedecanoate, which exhibit intermolecular excimer formation, have been widely used to study the physical and structural properties of lipid membranes [33,42,45–50]. These probes have been used to examine spontaneous interbilayer lipid transfer [46], protein-mediated interbilayer lipid transfer [47], and phospholipid lateral phase separation [48] as well as lateral diffusion [42,45,49, 50].

Recent studies have, however, shown that, since intermolecular excimer formation involves a bimolecular mechanism and is, therefore, concentration-dependent [51], difficulties may arise, particularly below a membrane's phase transition temperature, due to aggregation of probe molecules [52]. In the present studies, the concentration of pyrenedecanoate in the preparations examined was carefully monitored. Moreover, steady-state fluorescence polarization studies using 12-AS, a fluorophore that, unlike pyrenedecanoate, does not have any differential partition in the membrane plane [43], confirmed the findings noted with pyrenedecanoate, i.e., that hypothyroid membranes were more fluid than their control counterparts.

Earlier studies, using the probe 1,6-diphenyl-1,3,5-hexatriene [15], demonstrated the existence of a lipid thermotropic transition in rat colonic apical membranes and their liposomes at 21–23° C. In basic agreement with these former studies, the present experiments, using pyrenedecanoate, demonstrated a thermotropic transition in control and hypothyroid rat apical colonic membranes at 19–21° C. Although these temperatures are well below 37° C, a physiological role for this alteration in the physical state of the lipid is not necessarily precluded, since differential scanning calorimetric studies have revealed that techniques using fluorescent probes detect only the lower critical temperature of broad transitions observed in rat enterocyte [37] and hepatic [53] plasma membranes. In the present studies, the transition temperatures detected in rat apical plasma membranes therefore probably represent the lower critical temperature of the transition.

Finally, it should be noted that quantitative changes in the fluidity of rat colonic apical plasma membranes similar to those detected in the present studies have been shown previously by our laboratory to influence a number of enzymatic activities and transport processes in these membranes [14–20]. It therefore seems reasonable to suggest that the membrane fluidity changes induced by hypothyroidism in the present experiments may be physiologically important. Further studies should clarify questions concerning the mechanism(s) responsible for alterations in the lipid composition and fluidity of colonic apical plasma membranes and their functional significance in hypothyroid animals.

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