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Hormonal Effects on Fatty Acid Binding and Physical Properties of Rat Liver Plasma Membranes

Friedhelm Schroeder
Department of Pharmacology, University of Missouri-Columbia School of Medicine, Columbia, Missouri 65212

Summary. The fluorescent fatty acids, *trans*-parinaric and *cis*-parinaric acid, were used as analogs of saturated and unsaturated fatty acids in order to evaluate binding of fatty acids to liver plasma membranes isolated from normal fed rats. Insulin (10^{-8} to 10^{-6} M) decreased *trans*-parinaric acid binding 7 to 26% while *cis*-parinaric acid binding was unaffected. Glucagon (10^{-6} M) increased *trans*-parinaric acid binding 44%. The fluorescence polarization of *trans*-parinarate, *cis*-parinarate and 1,6-diphenyl-1,3,5-hexatriene was used to investigate effects of triiodothyronine, insulin and glucagon on the structure of liver plasma membranes from normal fed rats or from rats treated with triiodothyronine or propylthiouracil. The fluorescence polarization of *trans*-parinarate, *cis*-parinarate, and 1,6-diphenyl-1,3,5-hexatriene was 0.300 ± 0.004, 0.251 ± 0.003, and 0.302 ± 0.003, respectively, in liver plasma membranes from control rats and 0.316 ± 0.003, 0.276 ± 0.003 and 0.316 ± 0.003, respectively, in liver plasma membranes from hyperthyroid rats ($p < 0.025$, $n = 5$). Propylthiouracil treatment did not significantly alter the fluorescence polarization of these probe molecules in the liver plasma membranes. Thus, liver plasma membranes from hyperthyroid animals appear to be more rigid than those of control animals. The effects of triiodothyronine, insulin and glucagon added *in vitro* to isolated liver plasma membrane preparations were also evaluated as follows: insulin (10^{-10} M) and triiodothyronine (10^{-10} M) increased fluorescence polarization of *trans*-parinaric acid, *cis*-parinaric acid and 1,6-diphenyl-1,3,5-hexatriene in liver plasma membranes while glucagon (10^{-10} M) had no effects. These hormonal effects on probe fluorescence polarization in liver plasma membranes were abolished by pretreatment of the rats for 7 days with triiodothyronine. Administration of triiodothyronine (10^{-10} M) *in vitro* increased the fluorescence polarization of trans-parinaric acid in liver plasma membranes from propylthiouracil-treated rats. Thus, hyperthyroidism appeared to abolish the *in vitro* increase in polarization of probe molecules in the liver plasma membranes. Temperature dependencies in Arrhenius plots of absorption-corrected fluorescence and fluorescence polarization of *trans*-parinaric acid, *cis*-parinaric acid and 1,6-diphenyl-1,3,5-hexatriene were noted near 25 °C in liver plasma membranes from triiodothyronine-treated rats and near 18 °C in liver plasma membranes from propylthiouracil-treated rats. In summary, hormones such as triiodothyronine, insulin and glucagon may at least in part exert their biological effects on metabolism by altering the structure of the liver plasma membranes.

Key words Liver · plasma membrane · triiodothyronine · propylthiouracil · insulin · fluorescence · fatty acid

Introduction

The effects of hormones such as insulin, glucagon, estrogen and thyroid hormones on lipid metabolism (Shlatz & Marinetti, 1972; Andia-Waltenbaugh & Friedman, 1978; Czech, 1977; Keyes & Heimberg, 1979; Schrade, Woodside & Eaton, 1979) may be mediated both by direct or indirect actions. Direct effects may be due to intracellular redistribution of Ca^{++} (Shlatz & Marinetti, 1972; Andia-Waltenbaugh, Kimura, Wood, Divakaran & Friedman, 1978; Kiss, 1979; Luly & Shinitzky, 1979; Rahwan, Piascik & Witiak, 1979; Livingstone & Schachter, 1980). Insulin (10^{-8} to 10^{-6} M) decreased Ca^{++} binding to liver plasma membranes by 10 to 30%, while glucagon increased Ca^{++} binding 80–100% (Shlatz & Marinetti, 1972). Epinephrine and hydrocortisone also increased Ca^{++} binding (Shlatz & Marinetti, 1972). As shown elsewhere, the uptake of fatty acids or fluorescent fatty acid analogs by hepatocytes, liver plasma membranes or KB cells is modulated by Ca^{++} (Moskal, Emaus & Holland, 1977; Soler-Argilaga, Russell, Werner & Heimberg, 1978[1,2]). Mg^{++} had no effect on fatty acid uptake. Another direct effect may be the alteration of fluidity and microviscosity of liver plasma membranes, microsomes and mitochondria (Kury, Ramwell & McConnell, 1974; Chen & Hoch, 1977; Davis, Kern, Showalter, Sutherland, Sinensky & Simon, 1978; Dipple & Houslay, 1978; Lee, Consiglio, Hobig, Dyer, Hardegee & Kohn 1978; Luly & Shinitzky, 1979). Thus, alteration of fatty acid uptake by a direct action of these hormones on liver and other membranes may be possible. The consequence of such an action may be a mechanism where-

[1] C. Soler-Argilaga, R.L. Russel, H.V. Werner, J. Billheimer, L.R. Forte and M. Heimberg (*in preparation*).
[2] F. Schroeder. 1982. *J. Biol. Chem.* (*submitted*).

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by membrane lipid and very low density lipoprotein (VLDL)[3] lipid fatty acid composition may be controlled via the diet as opposed to alteration of fatty acid synthetic pathways. Insulin (Nelson, 1980) and thyroid hormone (Hulbert, 1978) may also have indirect effects on cell membrane fluidity. These effects appear to be mediated via altered fatty acid composition of membrane phosphoglycerides (Peifer, 1968; Platner, Patnayak & Chaffee, 1972; Chen & Hoch, 1977; Faas & Carter, 1981) and can be accounted for at least in part by altered fatty acid desaturase enzymes (Faas & Carter, 1981) or altered secretion of very low density lipoproteins (Keyes & Heimberg, 1979; Schroeder, Wilcox, Keyes & Heimberg 1981). The experiments described in this report were designed to determine whether certain hormones such as insulin, glucagon or thyroid hormones interact with Ca^{++} to affect binding of fluorescent fatty acids to and physical properties of liver plasma membranes. *Trans*-parinaric and *cis*-parinaric acid are fluorescent analogs of saturated and unsaturated fatty acids. These fatty acids fluoresce only when bound to the liver plasma membranes and therefore binding can be determined simply by measurement of increases in absorption-corrected fluorescence.[2]

Materials and Methods

Materials

Trans-parinaric acid and *cis*-parinaric acid were obtained from Molecular Probes, Inc., Plano, TX. 1,6-diphenyl-1,3,5-hexatriene was purchased from Aldrich Chemical Co., Milwaukee, WI. 3,5,3-'triiodothyronine (sodium salt) and 6-N-propyl-2-thiouracil were obtained from Sigma Chemical Co., St. Louis, MO. Insulin and glucagon were a gift of Eli Lilly and Co., Indianapolis, IN.

Animal Treatments

Unless otherwise specified, male Sprague-Dawley C.R. rats (Charles River Breeding Labs, Wilmington, MA) weighing 200–300 g were fed a standard chow (Purina Laboratory Chow) *ad libitum* prior to sacrifice. Rats having initial body weights of 150–175 g were maintained and either untreated or injected subcutaneously daily for 7 days with either 0.9% saline, 10 µg triiodothyronine per 100 g body weight, or with 1 mg propylthiouracil per 100 g body weight (Keyes & Heimberg, 1979). Under these conditions, all groups consumed the same amount of food daily.

Liver Plasma Membrane Isolation

The liver plasma membranes were isolated by the method of Pohl (1976). Plasma membranes were removed from the sucrose gradient and stored at −70 °C. Prior to use, the membranes were thawed, diluted 1:10 with 0.05 M Tris-HCl, 0.1 M NaCl (pH 7.4) buffer, and centrifuged at 39,000 rpm on a type 40 Ti Beckman Rotor. The membranes were resuspended in 0.05 M Tris-HCl, 0.1 M NaCl (pH 7.4). Storage at −70 °C of the liver plasma membranes taken directly from the sucrose gradient did not alter the fatty acid or calcium binding properties of the plasma membrane. Protein was determined by the method of Lowry, Rosebrough, Farr and Randall (1951).

[3] Abbreviations used: VLDL, very low density lipoproteins; EGTA, ethyleneglycolbis (beta-aminoethyl ether) N,N′-tetracetic acid; BSA, bovine serum albumin.

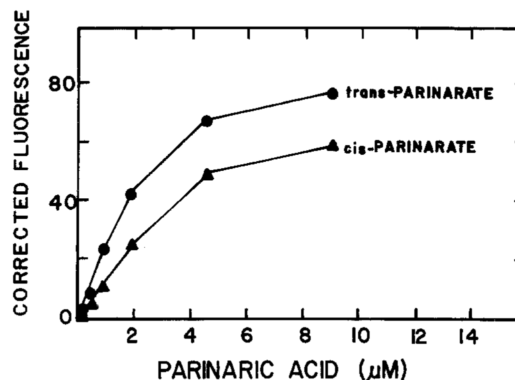

Fig. 1. Fatty acid binding by rat liver plasma membranes. Liver plasma membranes (50 µg protein/ml) were incubated at 24 °C in 0.05 M Tris-HCl, 0.1 M NaCl, pH 7.4, with increasing concentrations of *trans*- or *cis*-parinaric acid. Corrected fluorescence was measured at 24 °C as described in Materials and Methods

Fluorescence Measurements

All fluorescence measurements were made with a computer-centered spectrofluorimeter (Holland, Teets & Timnick, 1973; Christman, Crouch, Holland & Timnick, 1980) as described by Schroeder and Goh (1980). *Trans*-parinarate, *cis*-parinarate, and 1,6-diphenyl-1,3,5-hexatriene were incorporated into liver plasma membranes at probe to phospholipid molar ratios of 1:100 or less as described elsewhere (Schroeder, Holland & Vagelos, 1976; Schroeder & Goh, 1979, 1980). The fatty acids were dissolved in ethanol and 10 µl were added to the liver plasma membranes (50 µg protein/ml) in buffer. The diphenylhexatriene was dissolved in tetrahydrofuran and added (1 to 3 µl). Absorption-corrected fluorescence, corrected for instrumental as well as inner filter effects (Holland et al., 1973; Christman et al., 1980) and fluorescence polarization were measured as described previously (Schroeder & Goh, 1979, 1980). Light scattering was eliminated by using appropriate cutoff filters. *Trans*-parinaric acid, *cis*-parinaric acid, and 1,6-diphenyl-1,3,5-hexatriene were excited at 313, 320 and 362 nm, respectively; fluorescence emission was monitored at 415, 425 and 424 nm, respectively. Sample temperature was varied and monitored as described previously (Schroeder & Goh, 1980). Arrhenius plots were determined by the computer-centered spectrofluorimeter at every °C on ascending temperature scans from 10 to 45°. Each value represents 30–40 determinations taken per millisecond. Breakpoints were taken from the computer plots of log [corrected fluorescence] *vs.* $[°\text{Kelvin}]^{-1}$ and are accurate to $\pm 1°$.

Fatty Acid Binding

Fatty acid binding was measured as the increase in absorption-corrected fluorescence of *trans*- and *cis*-parinaric acid when these fatty acids were incubated with liver plasma membranes (50 µg Protein/ml buffer) at 24 °C unless otherwise stated. These fatty acids do not have a measurable fluorescence in aqueous buffers (Schroeder et al., 1976; Sklar, Hudson & Simoni, 1977; Schroeder & Goh, 1979, 1980; Sklar, Miljanich & Dratz, 1979). In contrast, when bound to liver plasma membranes both *trans*- and *cis*-parinaric acid fluoresce with high quantum yield. At 24 °C the binding, as measured by increasing fluorescence as a function

of time, was maximal in 3–5 min for trans-parinaric acid and 10–15 min for cis-parinaric acid. Incubation of liver plasma membranes for 15 min at 24 °C with increasing amounts of *trans*- or *cis*-parinaric acid resulted in saturable increases in fluorescence (Fig. 1). K_D's calculated from these data were 7.8 and 11.1 μM for *trans*- and *cis*-parinarate, respectively.

Results

Effects of Calcium, EGTA and Hormones on Parinaric Acid Fluorescence in Liver Plasma Membranes

Trans- and *cis*-parinaric acid are fluorescent analogs of saturated straight-chain and unsaturated kinked-chain fatty acids. The binding of these fatty acids may provide a suitable model system for comparing binding of saturated versus unsaturated fatty acids by liver plasma membranes. As shown in Fig. 2*A*, treatment of liver plasma membranes with 3.4 mM EGTA increased corrected fluorescence of *trans*-parinaric acid in liver plasma membranes. This increase was largely due to enhanced binding of the fatty acid.[2] Addition of 2.4 mM Ca^{++} decreased trans-parinarate-corrected fluorescence; this was not reversible with 3.4 mM EGTA (Fig. 2*B*). Addition of 10^{-8} M insulin gradually decreased *trans*-parinaric acid fluorescence in the liver plasma membrane from normal animals (Fig. 2*C* and Table 1). In addition, the insulin prevented the large increase in corrected fluorescence of trans-parinaric acid induced by 3.4 mM EGTA, as depicted in Fig. 2*A*. In contrast to insulin, 10^{-7} M glucagon increased the corrected fluorescence of *trans*-parinaric acid 10% (Table 1) and diminished but did not abolish the increase in fluorescence after addition of EGTA (Fig. 3). The increase in fluorescence was more marked at 10-fold higher glucagon concentration, 10^{-6} M (Table 1). Insulin (10^{-8} M) did not affect the fluorescence of the *cis*-parinaric acid and decreased fluorescence after addition of EGTA (Fig. 4 and Table 1). In comparison, EGTA alone in the absence of insulin increased *cis*-parinaric acid fluorescence.[2] Thus, insulin and glucagon had opposite effects on *trans*-parinarate fluorescence in the liver plasma membranes just as they have opposing effects on lipid and carbohydrate metabolism. Insulin also grossly altered the response of the membrane to EGTA. The effect on fatty acid binding may therefore be mediated via calcium. In addition, insulin decreased the *trans*-parinaric acid fluorescence but had little effect on the *cis*-parinaric acid fluorescence. The hormone may therefore have differential effects on binding of saturated versus unsaturated fatty acids.

Effects of addition of Ca^{++} instead of EGTA on the fluorescence of *trans*- and *cis*-parinarate in the presence of either insulin or glucagon are shown in Fig. 5. Addition of calcium decreased the fluorescence of *trans*-parinarate more rapidly in the presence of insulin than in the absence of insulin (Fig. 5*A*). Calcium in the presence of glucagon caused a slower decrease in *trans*-parinaric acid fluorescence in liver plasma membranes (Fig. 5*C*). The effects of calcium plus insulin on *cis*-parinaric acid fluorescence (Fig. 5*B*) were opposite to those of *trans*-parinaric

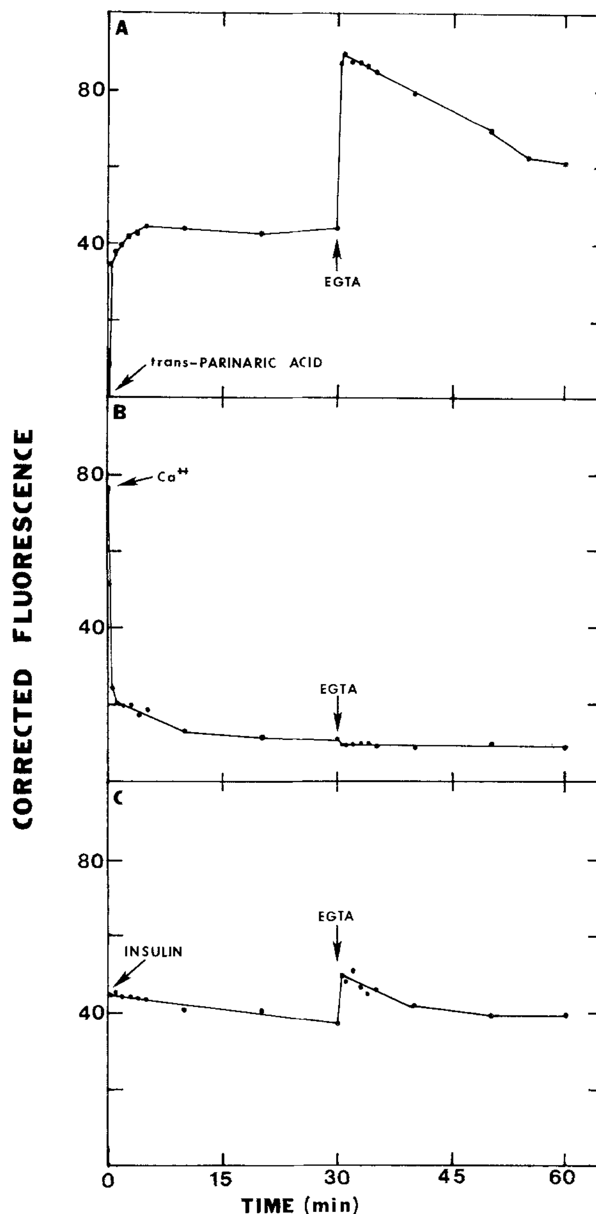

Fig. 2. Effects of insulin on binding of *trans*-parinaric acid by liver plasma membranes. Liver plasma membranes (50 μg/ml) were incubated at 24 °C in 0.05 M Tris-HCl, 0.1 M NaCl, pH 7.4, as described in Materials and Methods. In panel *A*, 2.4 mM Ca^{++} was also present; at time zero trans-parinaric acid was added and at 30 min, 3.4 mM EGTA was added. In panel *B*, 2.4 mM Ca^{++} was added at time zero and at 30 min 1 mM EGTA was added. In panel *C*, 2.4 mM Ca^{++} was present and 10^{-8} M insulin was added at time zero; 3.4 mM EGTA was added at 30 min. In *B* and *C* the *trans*-parinaric acid was added 5 min prior to zero time

Table 1. Effects of insulin and glucagon on *trans*-parinarate and *cis*-parinarate fluorescence in liver plasma membranes

Probe	Hormone (μM)	% Change in corrected fluorescence[a]
Trans-parinarate	10^{-8} insulin	-7 ± 3
	10^{-7} insulin	-11 ± 4*
	10^{-6} insulin	-26 ± 4*
Cis-parinarate	10^{-8} insulin	0 ± 1
	10^{-7} insulin	0 ± 1
	10^{-6} insulin	$+5 \pm 3$
Trans-parinarate	10^{-7} glucagon	$+10 \pm 1$*
	10^{-6} glucagon	$+44 \pm 4$*

[a] Liver plasma membranes were preincubated for 10 min at 37 °C in 0.15 M Tris buffer, pH 7.4, containing 1 mM Ca^{++}, and 1.8 μM *trans*-parinarate or *cis*-parinarate. The fluorescence was determined before addition of hormone and after incubation for 4 min at 24 °C with hormone. Values represent the mean ± SEM ($n=5$ animals). An asterisk denotes $p<0.01$ (compared to control values) by student's t test.

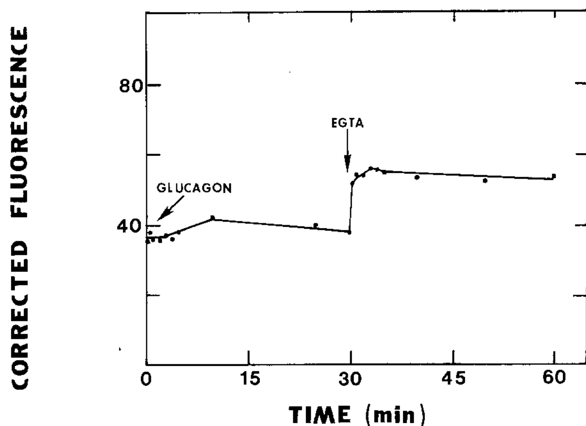

Fig. 3. Effects of glucagon on binding of *trans*-parinaric acid by liver plasma membranes. Experimental conditions were described in the legend to Fig. 2*C*, except that 10^{-7} M glucagon was added at zero time instead of insulin

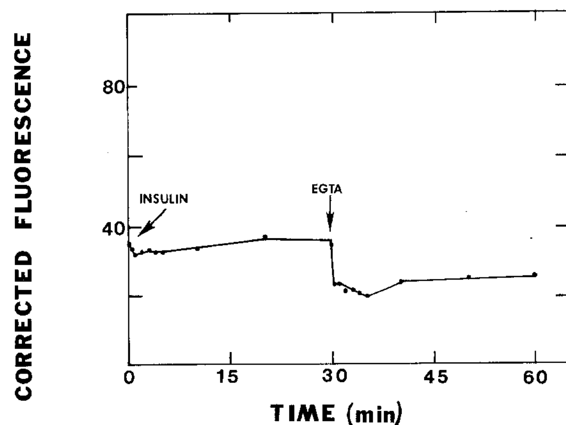

Fig. 4. Effects of insulin on binding of *cis*-parinaric acid by liver plasma membranes. Experimental conditions were as described in the legend to Fig. 2*C*, except that *cis*-parinaric acid was used instead of *trans*-parinaric acid

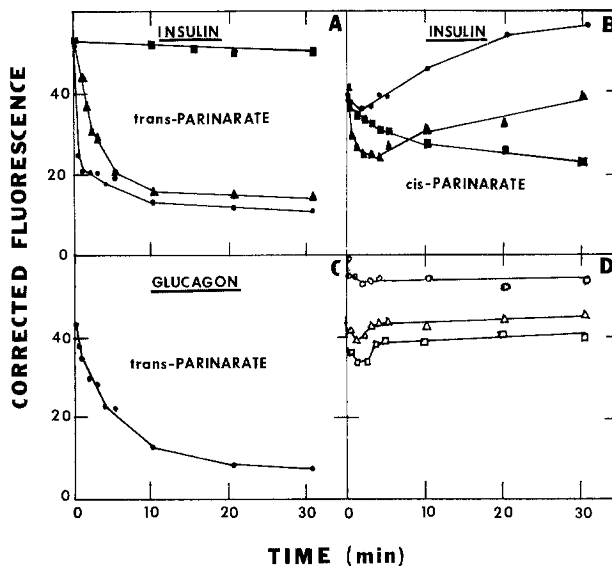

Fig. 5. Effects of insulin and glucagon on binding of *trans*- and *cis*-parinaric acid by liver plasma membranes. As seen in panels *A*, *B* and *C*, liver plasma membranes were incubated in Tris buffer, pH 7.2 at 37 °C for 15 min in the presence of either 10^{-8} M insulin or 10^{-7} M glucagon followed by addition of 1 mM Ca^{++} (●——●), or presence of no hormone followed by addition of 1 mM Ca^{++} (▲——▲), or presence of no hormone and no additions (■——■). *Trans*- or *cis*-parinarate were added at time zero. In *D*, the *trans*-parinaric acid was incubated with liver plasma membranes at 37 °C for 15 min in Tris buffer without Ca^{++} (pH 7.2) followed by addition of 10^{-8} M insulin (○——○), or 10^{-7} M glucagon (□——□); similarly *cis*-parinaric acid was incubated followed by addition of 10^{-8} M insulin (△——△)

acid in the plasma membranes (Fig. 5*A*); Ca^{++} plus insulin enhanced *cis*-parinaric acid fluorescence but decreased *trans*-parinaric acid fluorescence. The effects of either insulin or glucagon on the parinaric acid fluorescence were much smaller when the hormones were added after the *trans*- or *cis*-parinaric acid had been incorporated into the membranes (Fig. 5*D*) rather than during incorporation (Fig. 5*A*, *B*, *C*). Only transient decreases in corrected fluorescence of *trans*- and *cis*-parinarate preincorporated into the liver plasma membranes were noted. Thus, the effects observed when the probes were already incorporated into the membrane may indicate that these hormones do not alter the binding of fatty acid that is already taken up by the liver plasma membranes while they do alter the association of additional free fatty acid to the plasma membranes. The reasons for this difference and the mechanisms whereby they occur are not known. It is possible that insulin and glucagon may mediate or modify *trans*- and *cis*-parinaric acid fluorescence in the plasma membrane in response to Ca^{++} but not in the absence of Ca^{++}. Elsewhere we have reported that Ca^{++} decreased the fluorescence of both of these fatty acids.[2]

Table 2. *In vitro* drug effects on fluorescence polarization of probe molecules in isolated liver plasma membrane[a]

Drug treatment	Drug concentration (M)	Polarization		
		Trans-Parinarate	*Cis*-Parinarate	1,6-diphenyl-1,3,5-hexatriene
Control	none	0.300 ± 0.003	0.251 ± 0.003	0.302 ± 0.004
Triiodothyronine	0.66×10^{-10}	0.323 ± 0.005**	0.260 ± 0.007	0.323 ± 0.007*
Triiodothyronine	0.66×10^{-8}	0.316 ± 0.004*	0.265 ± 0.005	0.318 ± 0.004*
Triiodothyronine	0.66×10^{-6}	0.318 ± 0.004**	0.272 ± 0.005**	0.314 ± 0.010
Propylthiouracil	0.66×10^{-10}	0.309 ± 0.008	0.261 ± 0.007	0.316 ± 0.008
Propylthiouracil	0.66×10^{-8}	0.301 ± 0.004	0.255 ± 0.005	0.318 ± 0.007
Propylthiouracil	0.66×10^{-6}	0.309 ± 0.008	0.256 ± 0.006	0.319 ± 0.011
Insulin	0.66×10^{-10}	0.314 ± 0.003**	0.258 ± 0.004	0.320 ± 0.004**
Insulin	0.66×10^{-8}	0.316 ± 0.004*	0.272 ± 0.006*	0.311 ± 0.006
Insulin	0.66×10^{-6}	0.314 ± 0.003**	0.283 ± 0.007**	0.315 ± 0.005*
Glucagon	0.66×10^{-10}	0.311 ± 0.006	0.271 ± 0.010	0.315 ± 0.002
Glucagon	0.66×10^{-8}	0.302 ± 0.003	0.257 ± 0.006	0.320 ± 0.005
Glucagon	0.66×10^{-6}	0.307 ± 0.005	0.258 ± 0.008	0.316 ± 0.003

[a] All methods as described in legend of Table 1 except that animals were not pretreated with drugs. Isolated liver plasma membranes were incubated with drug + fluorescence probe molecules at 37 °C for 30 min in buffer containing no added Ca^{++} or EGTA. Polarization was determined at 24 °C as described in Materials and Methods. Values represent mean ± SEM ($n = 6$ animals). An asterisk refers to $p < 0.025$ while a double asterisk refers to $p < 0.01$ compared to the control values.

Effects of Hormones on Fluorescence Polarization of Probe Molecules in Liver Plasma Membrane

It is possible that the hormonal effects on corrected fluorescence were due to alteration in liver plasma membrane rigidity. The probe concentration independent parameter, polarization, can be used as a relative measure of rigidity. If the fluorescence lifetime of the probe molecules in the membranes is determined, the polarization value can be converted to microviscosity and rotational correlation time of the probe. Both parameters provide good measures of the rigidity of the microenvironment in which the probe molecules reside. The data in Table 2 indicate that insulin increased the polarization of both *trans*-parinaric acid and *cis*-parinaric adid. Glucagon had no such action. It is known that trans-parinaric acid partitions preferentially into rigid or 'solid' lipid regions while *cis*-parinarate partitions more equally between fluid and solid lipid (Sklar et al. 1977). Thus, the more rigid areas of membrane detected by *trans*-parinaric acid appeared to become more rigid after addition of insulin, while the fluid areas sensed by *cis*-parinaric acid did not. This interpretation may be complicated by effects of calcium on trans-parinaric acid which might alter polarization values (Sklar et al. 1979). However, the polarization of 1,6-diphenyl-1,3,5-hexatriene, a probe molecule without ionizable side groups, was also increased by addition of insulin (Table 2). Similarly, membrane from rats treated with triiodothyronine but not propylthiouracil increased the polarization of *trans*- and *cis*-parinaric acid. Treatment of the liver plasma membrane with 3.4 mM EGTA (*data not shown*) abolished the insulin effect on *trans*- and *cis*-parinarate fluorescence polarization.

The effects of insulin and glucagon on fluorescence polarization of probe molecules in plasma membranes from liver of rats pretreated for 7 days molecules in plasma membranes from liver of rats pretreated for 7 days with triiodothyronine was also determined (Table 3). The polarization of *trans*-parinarate, *cis*-parinarate, and 1,6-diphenyl-1,3,5-hexatriene appeared to be increased in liver plasma membranes from triiodothyronine-treated rats (T_3-treated *vs.* untreated controls). Addition of insulin, glucagon, propylthiouracil or additional triiodothyronine *in vitro* had no further effect. Thus, treatment *in vivo* with triiodothyronine appeared to increase the rigidity of the isolated liver plasma membranes but made them refractile to any changes by *in vitro* addition of insulin or glucagon.

Prior treatment of the rat with propylthiouracil was insufficient to alter the polarization of the *trans*- and *cis*-parinarate or 1,6-diphenyl-1,3,5-hexatriene in the isolated liver plasma membranes (Table 4). Addition *in vitro* of triiodothyronine, insulin or glucagon to membranes from propylthiouracil-treated rats increased the fluorescence polarization of *trans*- and *cis*-parinarate but not of 1,6-diphenyl-1,3,5-hexatriene. Addition of propylthiouracil *in vitro* did not affect the fluorescence polarization of these probe molecules. The mild hypothyroid state produced by

Table 3. *In vitro* drug effects on fluorescence polarization of probe molecules in liver plasma membranes from rats pretreated with triiodothyronine[a]

Drug treatment	Drug concentration (M)	Polarization		
		Trans-Parinarate	*Cis*-Parinarate	1,6-diphenyl-1,3,5-hexatriene
Control (no treatment)	none	0.300±0.003	0.251±0.003	0.302±0.004
Control (T_3-treated)	none	0.316±0.003*	0.276±0.003*	0.316±0.003*
Triiodothyronine	0.66×10^{-10}	0.310±0.006	0.282±0.008	0.325±0.007
Triiodothyronine	0.66×10^{-8}	0.319±0.002	0.286±0.009	0.302±0.008
Triiodothyronine	0.66×10^{-6}	0.318±0.005	0.290±0.010	0.318±0.003
Propylthiouracil	0.66×10^{-10}	0.304±0.008	0.290±0.010	0.314±0.005
Propylthiouracil	0.66×10^{-8}	0.302±0.007	0.282±0.008	0.311±0.006
Propylthiouracil	0.66×10^{-6}	0.311±0.010	0.266±0.008	0.313±0.004
Insulin	0.66×10^{-10}	0.308±0.008	0.289±0.009	0.318±0.008
Insulin	0.66×10^{-8}	0.312±0.004	0.295±0.012	0.306±0.010
Insulin	0.66×10^{-6}	0.315±0.002	0.282±0.008	0.318±0.004
Glucagon	0.66×10^{-10}	0.314±0.006	0.274±0.008	0.316±0.002
Glucagon	0.66×10^{-8}	0.306±0.006	0.258±0.006	0.309±0.007
Glucagon	0.66×10^{-6}	0.312±0.008	0.268±0.007	0.313±0.004

[a] All conditions as described in legend of Table 1 except that rats had been pretreated with triiodothyronine as described in Materials and Methods. Values represent the mean±SEM ($n=6$ animals). An asterisk refers to $p < 0.01$ compared to control (no treatment). No significant differences between drug-treated and control (T_3-treated) groups were noted.

Table 4. *In vitro* drug effects on fluorescence polarization of probe molecules in liver plasma membranes from rats pretreated with propylthiouracil

Drug treatment	Drug concentration (M)	Polarization		
		Trans-Parinarate	*Cis*-Parinarate	1,6-diphenyl-1,3,5-hexatriene
Control (untreated)	none	0.300±0.003	0.251±0.003	0.302±0.004
Control (PTU-treated)	none	0.309±0.004	0.260±0.006	0.315±0.008
Triiodothyronine	0.66×10^{-10}	0.331±0.008*	0.290±0.007**	0.310±0.005
Triiodothyronine	0.66×10^{-8}	0.333±0.007*	0.289±0.006**	0.298±0.009
Triiodothyronine	0.66×10^{-6}	0.323±0.006	0.286±0.006*	0.300±0.008
Propylthiouracil	0.66×10^{-10}	0.302±0.005	0.275±0.008	0.306±0.007
Propylthiouracil	0.66×10^{-8}	0.309±0.008	0.276±0.010	0.296±0.010
Propylthiouracil	0.66×10^{-6}	0.312±0.010	0.273±0.009	0.312±0.007
Insulin	0.66×10^{-10}	0.308±0.005	0.279±0.005	0.306±0.007
Insulin	0.66×10^{-8}	0.297±0.008	0.288±0.005**	0.305±0.004
Insulin	0.66×10^{-6}	0.332±0.006**	0.297±0.010**	0.314±0.003
Glucagon	0.66×10^{-10}	0.300±0.006	0.279±0.010	0.302±0.006
Glucagon	0.66×10^{-8}	0.332±0.009*	0.291±0.008*	0.313±0.004
Glucagon	0.66×10^{-6}	0.333±0.008*	0.293±0.009*	0.307±0.008

[a] All conditions as described in legend of Table 1 except that rats had been pretreated with triiodothyronine as described in Materials and Methods. Values represent the mean±SEM ($n=6$ animals). An asterisk refers to $p < 0.05$ as compared to control (PTU-treated) while a double asterisk refers to $p < 0.01$ compared to control (PTU-treated).

this treatment (Keyes & Heimberg, 1979) did not abolish the polarization response of probe molecules in the liver plasma membranes to insulin or glucagon.

It is possible that the effects of these hormones may be mediated in part through the action of calcium. The effects of Ca^{++} and EGTA on fluorescence polarization of liver plasma membranes from rats pretreated with triiodothyronine or propylthiouracil are illustrated in Table 5. With the exception of diphenylhexatriene the polarization values were higher in the presence of calcium than in the presence of EGTA. The addition of EGTA abolished the effects of pretreatment with triiodothyronine or propylthiouracil on fluorescence polarization of trans-parinarate. In contrast, in the presence of calcium, the fluorescence polarization of trans-parinarate in plasma membranes

Table 5. Effect of pretreatment of rats with drugs on fluorescence polarization of probe molecules in isolated liver plasma membranes[a]

Drug treatment	Buffer (mM)		Polarization		
	Ca^{++}	EGTA	*Trans*-Parinarate	*Cis*-Parinarate	1,6-diphenyl-1,3,5-hexatriene
Control	2.4	0	0.323±0.007**	0.275±0.010*	0.299±0.006
Control	0	1	0.288±0.005	0.249±0.006	0.283±0.006
Control	3.4	1	0.320±0.006**	0.290±0.008**	0.294±0.005
Triiodothyronine	2.4	0	0.319±0.008**	0.263±0.009*	0.304±0.003
Triiodothyronine	0	1	0.290±0.003	0.236±0.008	0.301±0.008
Triiodothyronine	3.4	1	0.318±0.003**	0.281±0.012**	0.316±0.007
Propylthiouracil	2.4	0	0.304±0.003*	0.252±0.011	0.306±0.008
Propylthiouracil	0	1	0.283±0.007	0.245±0.009	0.296±0.006
Propylthiouracil	3.4	1	0.305±0.006*	0.301±0.012*	0.304±0.010

[a] Rats were pretreated with saline (control), triiodothyronine, or propylthiouracil as described in Materials and Methods. Liver plasma membranes were resuspended in 0.050 M Tris, 0.1 M NaCl, pH 7.5 plus $CaCl_2$ or EGTA at 50 µl/ml and incubated for 30 min at 37 °C. Polarization was determined at 24 °C as described in Materials and Methods. Values represent the mean±SEM ($n=3$ animals). An asterisk refers to $p<0.05$ while a double asterisk refers to $p<0.01$ as compared to 0 Ca^{++}, 1 mM EGTA within each treatment group.

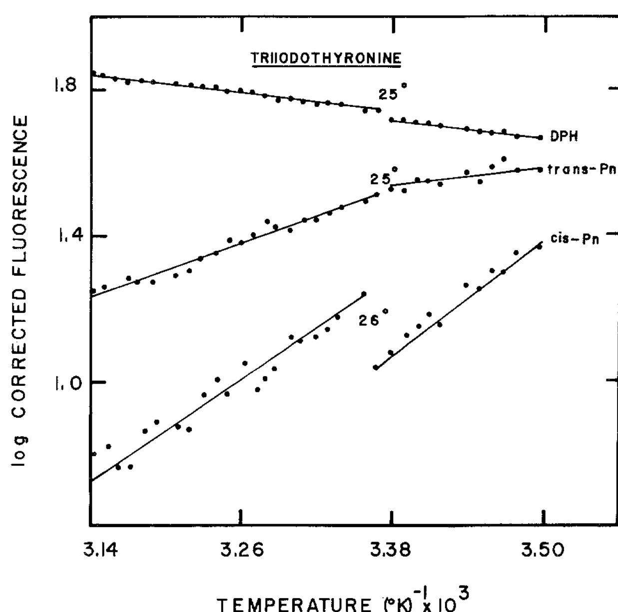

Fig. 6. Temperature dependence of corrected fluorescence in liver plasma membranes from triiodothyronine-treated rats. *Cis*-parinaric acid (*cis*-Pn), trans-parinaric acid (*trans*-Pn), and 1,6-diphenyl-1,3,5-hexatriene were incorporated into liver plasma membranes (50 µg protein/ml) in 0.05 M Tris HCl-0.1 M NaCl, pH 7.4, at 37 °C for 15 min, as described in Materials and Methods. The plasma membranes were prepared from rats treated with triiodothyronine

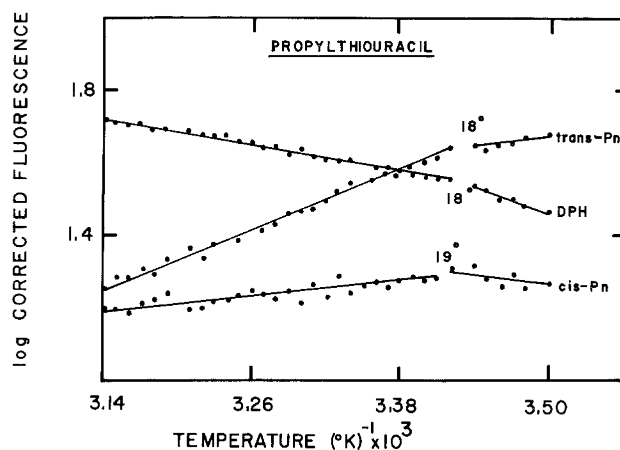

Fig. 7. Temperature dependence of corrected fluorescence in liver plasma membranes from propylthiouracil-treated rats. Experimental conditions were identical to those described in the legend to Fig. 6 except that the rats were treated with propylthiouracil as described earlier

from propylthiouracil, but not triiodothyronine, -pretreated rats was lower than control values. *Cis*-parinarate and 1,6-diphenyl-1,3,5-hexatriene fluorescence polarization values were basically similar to corresponding control values. EGTA treatment also abolished the effects of insulin on *trans*-parinarate fluorescence in the liver plasma membranes (*data not shown*). In summary, fluorescence polarization of *trans*-parinarate, *cis*-parinarate, and 1,6-diphenyl-1,3,5-hexatriene in liver plasma membrane varied with prior treatment of the animal with triiodothyronine or propylthiouracil, the addition of insulin or glucagon to the membrane preparation *in vitro*, and on the presence or absence of Ca^{++} and/or EGTA in the buffer system.

Temperature Dependence of Fluorescence Probes in Liver Plasma Membranes of Hormone-Treated Rats

The effects of treatment with triiodothyronine of the rats on the temperature dependence of probe molecules in the liver plasma membranes is shown in Fig. 6. A characteristic breakpoint near 25 °C was noted in the Arrhenius plots of corrected fluorescence

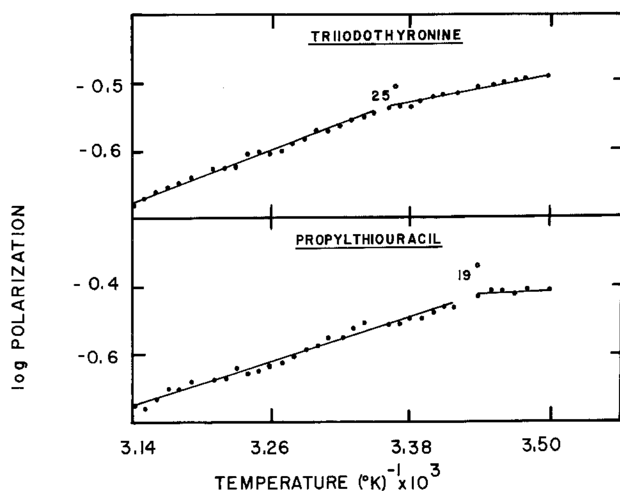

Fig. 8. Temperature dependence of 1,6-diphenyl-1,3,5-hexatriene fluorescence polarization in liver plasma membranes from triiodothyronine- and propylthiouracil-treated rats. Experimental conditions were identical to those described in the legends to Figs. 6 and 7

of *trans*-parinaric acid, *cis*-parinaric acid, and 1,6-diphenyl-1,3,5-hexatriene in the liver plasma membrane from hyperthyroid rats. In contrast, these same fluorescence molecules showed a temperature dependency near 18 °C in liver plasma membranes from propylthiouracil-treated rats (Fig. 7). These results were confirmed by Arrhenius plots of 1,6-diphenyl-1,3,5-hexatriene fluorescence polarization (Fig. 8). Liver plasma membranes from untreated rats had characteristic temperatures near 21 and 31 °C (*data not shown*). Thus, triiodothyronine treatment raised while propylthiouracil treatment lowered the characteristic breakpoints in temperature dependencies of fluorescent probes in the liver plasma membranes.

Discussion

Uptake of long-chain fatty acids has been believed to follow their spontaneous dissociation from albumin in the bulk aqueous phase, with subsequent diffusion of the free ligand to the surface of the liver cell, although a recent report indicates that a saturable interaction of the fatty acid-albumin complex with a receptor for albumin on the liver cell surface may occur (Weisiger, Gollan & Ockner, 1981). Such a receptor may account for the efficient extraction of fatty acids by the liver. In either case, the free fatty acid is taken up. In the system used herein, the free fatty acid uptake alone was investigated in the absence of serum albumin mediation. The presence of serum albumin would have interfered with the analysis as well since BSA bound *trans*- and *cis*-parinaric acid are highly fluorescent (Sklar et al. 1977, 1979).

The data presented here demonstrate a potential role of insulin and glucagon in fatty acid binding by liver plasma membranes. The two hormones had opposite effects on binding of *trans*-parinaric acid by liver plasma membranes, as measured by absorption-corrected fluorescence. In contrast, insulin had little effect on the *cis*-parinaric acid fluorescence. Thus, insulin may differentially alter the binding of saturated (or *trans*- double bonded) versus unsaturated (or *cis*- double bonded) fatty acids. Permeation of fatty acids across intestinal and Ehrlich ascites cell membranes increased linearly with increasing chain length of the fatty acid and decreased with increasing number of double bonds (Spector & Fletcher, 1978; Salee, 1979). The effects of insulin and glucagon appear to be related to Ca^{++} levels, since insulin and glucagon modulated the effect of EGTA on binding of *trans*-parinaric acid and reversed the effect of EGTA on binding of *cis*-parinaric acid. Insulin decreased while glucagon increased Ca^{++} binding to rat liver plasma membranes (Shlatz & Marinetti, 1972). Ca^{++} decreased the binding of trans-parinaric acid[2]. Therefore, if the effect of insulin or *trans*-parinaric acid binding is mediated exclusively through Ca^{++}, then insulin should increase *trans*-parinaric acid binding while glucagon should decrease it. However, as shown in Table 1, the opposite occurred. Thus, alteration in Ca^{++} binding alone cannot be responsible for the effects of insulin or glucagon on fluorescent fatty acid binding by liver plasma membranes. A recent report indicates that a receptor for albumin on the liver cell surface may mediate uptake of fatty acids (Weisiger et al., 1981). However, albumin was not present in our incubation mixtures. Another possibility is that fatty acid binding may be mediated through the effects of insulin on liver plasma membrane microviscosity and polarization of fluorescence probes. As reported here and elsewhere (Luly & Shinitzky, 1979; Livingstone & Schachter, 1980), insulin increased the polarization of fluorescence probe molecules in the liver plasma membranes. The resulting increases in rigidity of the membrane would tend to increase the quantum yield of fluorescence. Thus, insulin would be expected to increase the fluorescence of *trans*-parinaric acid if this mechanism were involved. The present data show that this was not the case. In summary, effects of insulin on fluorescent fatty acid binding did not appear to be mediated through the small decrease in Ca^{++} binding (10 to 30%) to the liver plasma membrane (Shlatz & Marinetti, 1972) or to the small increases (10%) in membrane rigidity (Luly & Shinitzky, 1979; Livingstone & Schachter, 1980). In fact, the decreases in Ca^{++} binding would be expected to decrease membrane rigidity rather than increase it. Insulin must

alter the fluorescent fatty acid binding by some other mechanism. It should be emphasized that prior treatment of the rat with triiodothyronine abolished the increased fluorescence polarization of *trans*-parinaric acid after addition *in vitro* of insulin normally seen in membranes from euthyroid rats. It is apparent, therefore, that the hormonal state of the rat prior to isolation of liver plasma membranes can modify the response of the membranes to added insulin, glucagon or triiodothyronine. Prior treatment of the rat with thyroid hormone increased the fluorescence polarization of *trans*- and *cis*-parinaric acid as well as 1,6-diphenyl-1,3,5-hexatriene. These results are consistent with the effects of triiodothyronine on red blood cell membranes (DeMendoza, Moreno, Massa, Morero & Farias, 1977).

Phase transitions in liver plasma membranes and thyroid cells membranes have been shown to correlate with changes in glucagon and epinephrine-stimulated adenylate cyclase activity (Kreiner, Keirns & Bitensky, 1973; Houslay, Metcalfe, Warren, Hesketh & Smith, 1976) or with thyroid-stimulating hormone binding to the gland (Mehdi, Nussey, Shindelman & Kriss, 1977). These transitions can be modified by lipid compositional alterations (Houslay, Hesketh, Smith, Warren & Metcalfe, 1976). Similarly, thyroidectomy of rats decreased transition temperatures of the liver mitochondrial membranes while thyroxine injections increased the transition temperature (Hulbert, Augee & Raison, 1976). This effect appears to be related to alterations in unsaturated/saturated fatty acid ratios in the mitochondrial membrane lipids (Steffen & Platner, 1976; Augee, Raison & Hulbert, 1979). Using three different fluorescence probe molecules and a fluorescence probe concentration-dependent parameter (corrected fluorescence) as well as a probe concentration-independent parameter (polarization), we observed that the characteristic temperature dependencies of liver plasma membranes from triiodothyronine-treated animals were near 25 °C while those of propylthiouracil-treated animals were near 18 °C.

In summary, the data presented here demonstrate the potential usefulness of using *trans*- and *cis*-parinaric acids as fluorescent fatty acid analogs to monitor the fatty acid binding and physical properties of isolated liver plasma membranes of control as well as triiodothyronine-treated animals.

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