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Studies on the mechanism of inhibition of nuclear triiodothyronine binding by fatty acids

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Studies were designed to elucidate the mechanism by which unsaturated fatty acids inhibit the binding of triiodothyronine (T3) ro rat liver nuclei. The possibility of a direct interaction between oleic acid and T3 was excluded by dialysis experiments. Oleic acid inhibits nuclear T3 binding in a strictly competitive manner. The K_i value of oleic acid was approx. 10^4 times greater than that of T3. The inhibitory effect of oleic acid could be reversed by bovine serum albumin.

Unsaturated fatty acid; Triiodothyronine; Nuclear triiodothyronine receptor; Albumin

1. INTRODUCTION

Now, there is considerable evidence that fatty acids have the capacity to modulate the binding of various ligands to their receptors [1,2]. We have recently demonstrated that unsaturated fatty acids are potent inhibitors of nuclear T3 binding [3]. The aim of the present study was to elucidate further the mechanism by which unsaturated fatty acids inhibit the binding of T3 to its nuclear receptor. Oleic acid was used as a representative fatty acid [3].

Firstly, a putative interaction between oleic acid and T3 was investigated using dialysis experiments. Secondly, the character of the inhibition of nuclear T3 binding to its receptor by oleic acid was studied. Finally, the reversibility of this inhibition was evaluated using bovine serum albumin as a fatty acid-binding protein.

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Abbreviations: T3, triiodothyronine; OA, oleic acid; BSA, bovine serum albumin

2. MATERIALS AND METHODS

2.1. *Chemicals*

Oleic acid was purchased from Sigma (St. Louis, USA), T3 from Henning GmbH (Berlin, Germany) and bovine serum albumin (BSA) from Calbiochem, Behring Diagnostica, Hoechst (fraction V fatty acid concentration $<0.01\%$, T3 and T4 concentration $<10^{-14}$ mol/mg BSA). Radioactive T3 was obtained from New England Nuclear, Boston, USA ([^{125}I]T3, spec. act. 2200 Ci/mmol).

2.2. *Equilibrium and symmetric dialysis*

In equilibrium dialysis, a two-compartment system separated by a semi-permeable membrane (Dianorm dialyser, Diachema AG, Zurich, Switzerland) was filled with incubation buffer (20 mM Tris-HCl, 0.25 M sucrose, 1 mM EDTA, 50 mM NaCl, 5% (v/v) glycerol, pH 7.6, solution B). A tracer dose of [^{125}I]T3 was injected into compartment 1. Equilibrium of tracer is reached when the ratio of counts in compartment 2 to compartment 1 reaches a value of 1.0. Incubation was at 22°C for periods of 2 and 18 h in the absence or presence of 0.33×10^{-4} M oleic acid (added to compartment 1).

The rate of [^{125}I]T3 diffusion was measured by symmetric dialysis, in which both compartments contained 0.33×10^{-4} M oleic acid in solution B. By drawing samples at various incubation times the diffusion rate constant of [^{125}I]T3 was calculated [4]. If $-\ln\{(C_1 - C_2)/(C_1 + C_2)\}$ is plotted versus diffusion time, the slope of the line will represent the diffusion rate constant (C_1 denotes the counts in compartment 1, C_2 the counts in compartment 2).

2.3. *Isolation of rat liver nuclei*

Livers of male Wistar rats were excised and stored in liquid

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Table 1

Symmetric dialysis of [^{125}I]T3 in the absence and presence of oleic acid

Incubation time (min)	No oleic acid		Oleic acid (0.33×10^{-4} M)	
	C_2/C_1	$-\ln\{(C_1-C_2)/(C_1+C_2)\}$	C_2/C_1	$-\ln\{(C_1-C_2)/(C_1+C_2)\}$
18	0.145	0.293	0.145	0.292
26	0.217	0.441	0.208	0.422
34	0.273	0.559	0.262	0.537
41	0.305	0.630	0.311	0.643
52	0.410	0.871	–	–
57	–	–	0.433	0.926

nitrogen until further processing. Nuclei were isolated at 4°C as described previously [5]. In short, the liver was homogenized in solution A (20 mM tricine, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5% (v/v) glycerol, 0.25 M sucrose, pH 7.6) and after three washes in solution A containing 0.5% Triton X-100 the remaining pellet was purified by centrifugation (45 min, 45 000 × g) in solution A containing 2.4 M sucrose. After two further washes with solution A + 0.5% Triton X-100, the isolated rat liver nuclei were suspended in 0.5 vol. (v/w) solution B.

2.4. *Nuclear binding of [^{125}I]T3*

0.1 ml freshly prepared nuclear suspension was incubated in solution B with 11–15 fmol [^{125}I]T3 in the presence of 5 mM dithiothreitol for 2 h at 22°C in a shaking water bath. Total volume was 0.5 ml. The incubation was stopped by chilling the samples on ice; thereafter the nuclei were pelleted (4°C, 1550 × g, 10 min) and washed twice with solution B + 0.5% Triton X-100. Specific binding was calculated by subtracting the radioactivity remaining with the nuclear pellet of parallel incubations containing an excess (10^{-7} M) of non-radioactive T3. The non-specific binding was 2.03 ± 0.24% of added [^{125}I]T3 and specific binding was 35.49 ± 1.69% (mean ± SE, $n = 20$).

To determine the association constant (K_a) and maximal binding capacity (MBC) of the binding of T3 to their nuclear receptors, Scatchard analysis was performed. To this end, increasing amounts of non-radioactive T3 (0–0.33 × 10^{-8} M) were added to the test tubes [6]. Scatchard analyses were also carried out in the presence of various concentrations of oleic acid, and in the presence of various extra amounts of non-radioactive T3 (which were not taken into account in the calculation of the Scatchard plot). The reversibility of the effect of oleic acid was determined by adding various concentrations of bovine serum albumin,

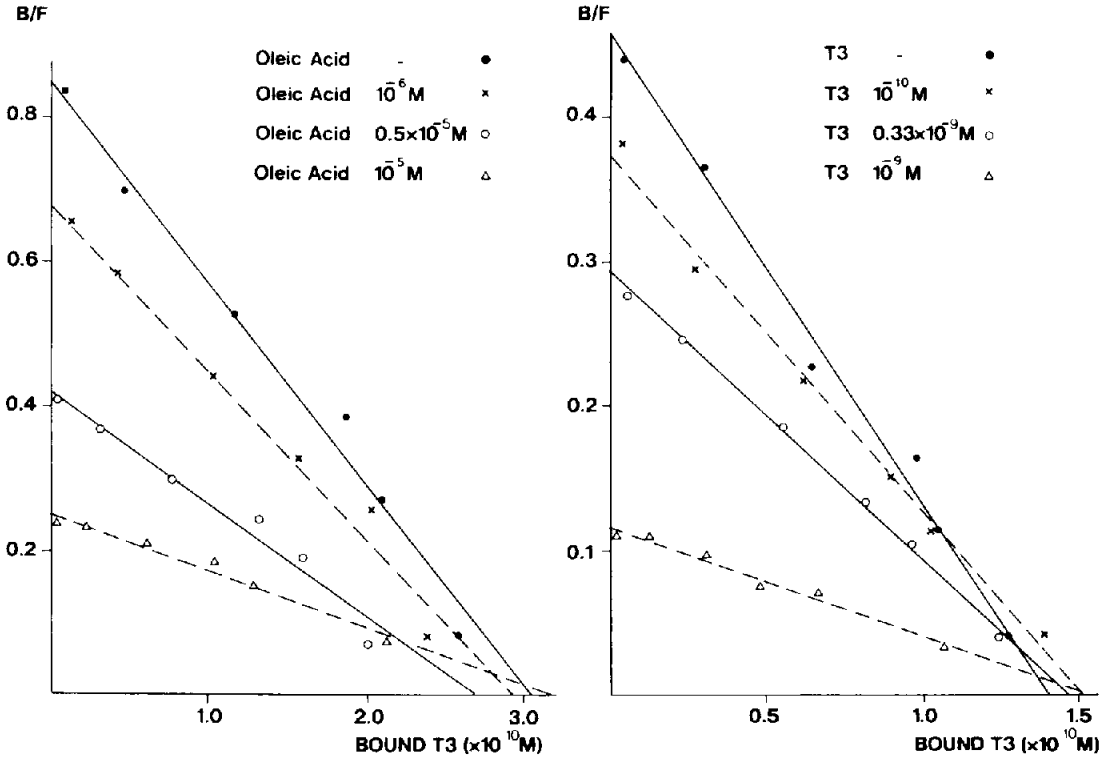

Fig.1. Scatchard plots of the binding of T3 to its nuclear receptor in the absence and presence of oleic acid (left panel) and T3 (right panel).

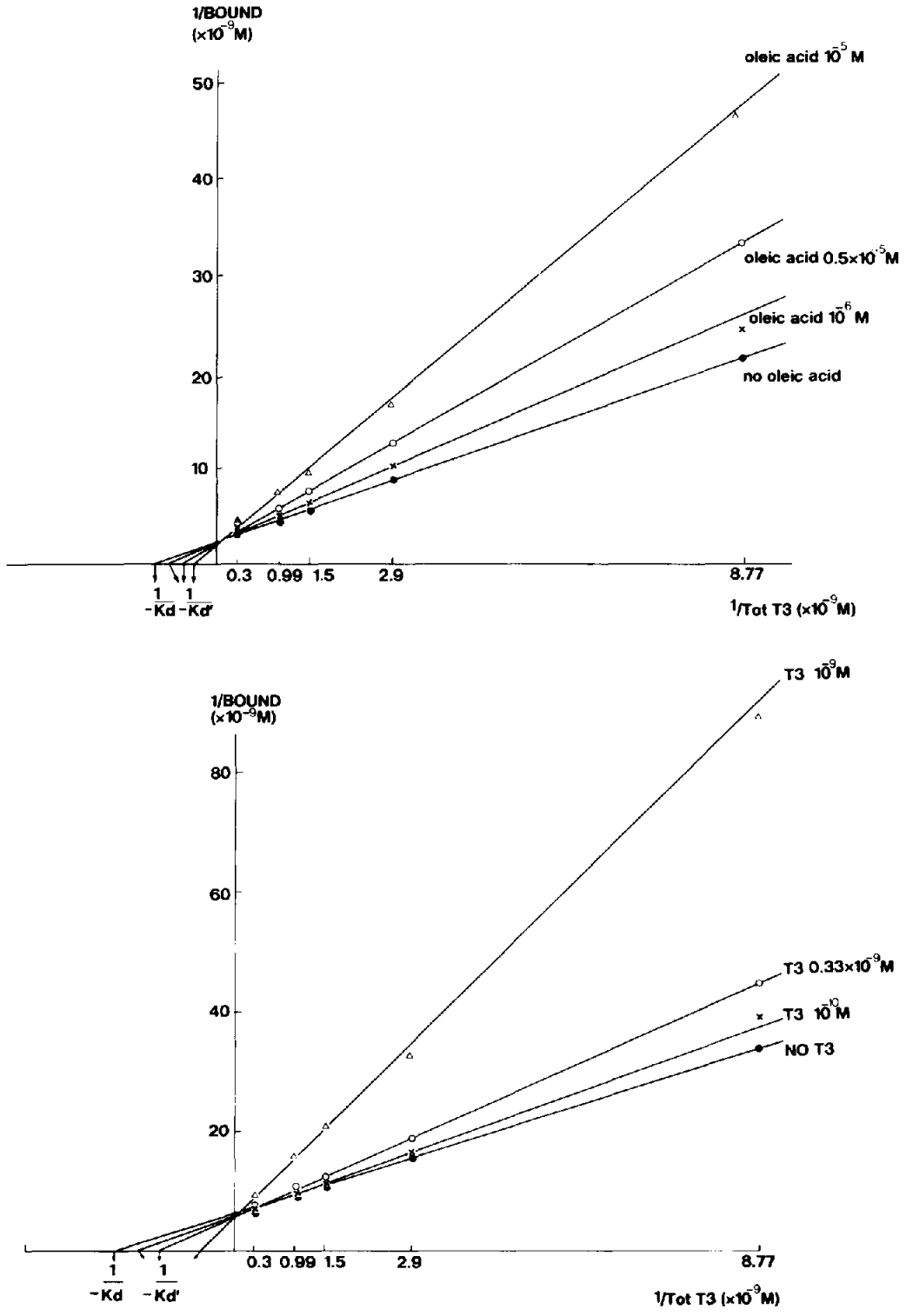

Fig.2. Lineweaver-Burk analysis of the effect of oleic acid (upper panel) and T3 (lower panel) on nuclear T3 binding.

dissolved in the incubation buffer, to the test tubes (final concentration 0.125, 0.25 and 0.5 mg/ml).

3. RESULTS

3.1. *Dialysis experiments: no binding of T3 to oleic acid*

In equilibrium dialysis, the ratio of [^{125}I]T3 in compartment 2 to [^{125}I]T3 in compartment 1 was 0.721 in the absence and 0.716 in the presence of oleic acid after 2 h incubation. Equilibrium was reached after 18 h; at that time the ratio of counts in the two compartments in the absence or presence of oleic acid was again similar (0.983 vs 0.990). The results of symmetric dialysis are shown in table 1. The diffusion rate constant of [^{125}I]T3 is 0.967 h^{-1} in the absence of oleic acid and 0.969 h^{-1} in the presence of oleic acid.

3.2. *Competitive inhibition of nuclear T3 binding by oleic acid*

Fig.1 depicts Scatchard plots of nuclear T3 binding in the absence and presence of oleic acid. Addition of increasing amounts of oleic acid results in a progressive decrease of apparent K_a whereas MBC remains unchanged. Similar results are obtained in the Scatchard plots with addition of various amounts of non-radioactive T3. Lineweaver-Burk analysis reveals a competitive inhibition of nuclear T3 binding by both oleic acid and T3 (fig.2). The inhibitor constant for oleic acid and T3 can be deduced from the next equation for competitive inhibition [7]:

$$K_{d'} = K_d(1 + i/K_i)$$

in which K_d is the dissociation constant for T3, $K_{d'}$ the apparent dissociation constant for T3 in the presence of inhibitor (T3 or oleic acid), i the concentration of inhibitor and K_i the dissociation constant for the inhibitor. If $K_{d'}$ is plotted versus i, the intercept of the x-axis will represent the $-K_i$ value. K_i is found to be 7.97×10^{-6} M for oleic acid and 3.78×10^{-10} M for T3 (fig.3).

3.3. *Reversibility of the inhibition of nuclear T3 binding by oleic acid*

Fig.4 depicts the time course of nuclear [^{125}I]T3 binding in the absence and presence of oleic acid. Dissociation of receptor bound [^{125}I]T3 is effectively obtained by delayed addition of an excess of either non-radioactive T3 or oleic acid. The effect of the addition of the two agents together on the dissociation of bound [^{125}I]T3 is greater than of each agent alone.

The effect of BSA on the inhibition of nuclear T3 binding by oleic acid is shown in fig.5. BSA by itself had no or minimal effect on the binding of [^{125}I]T3 in a concentration up to 0.5 mg/ml. BSA

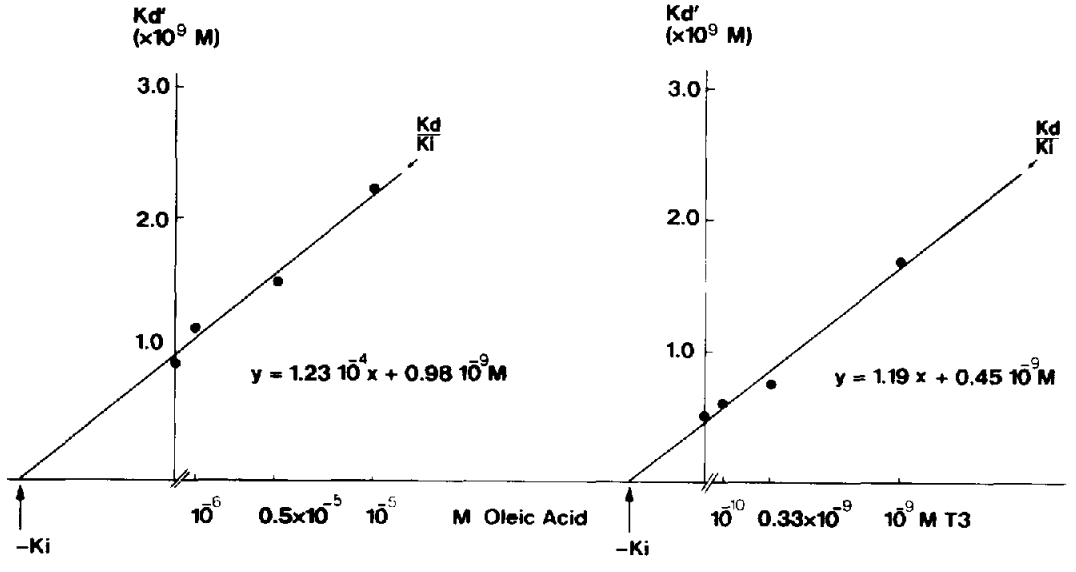

Fig.3. Graphical determination of inhibitor constant for oleic acid (left panel) and for T3 (right panel).

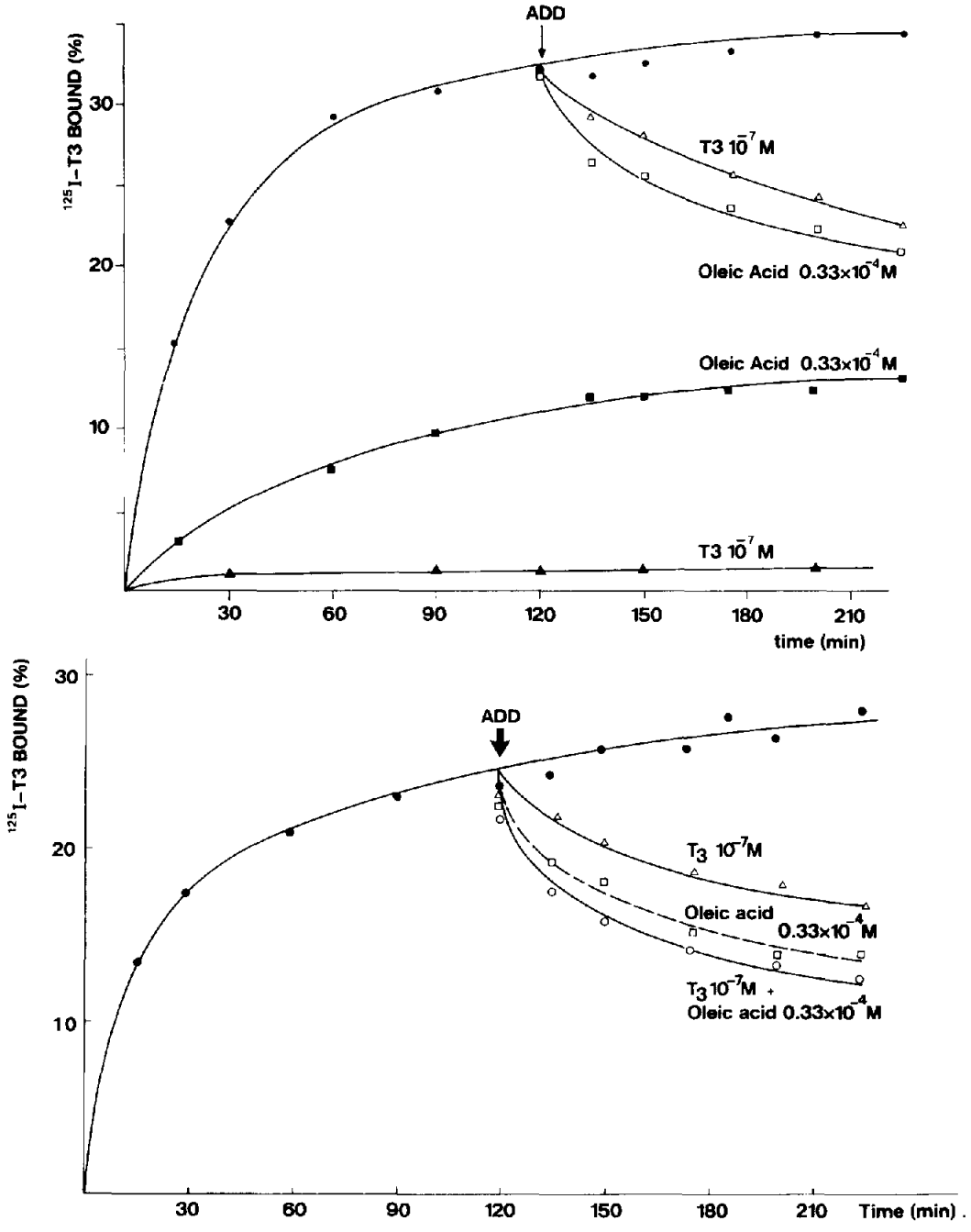

Fig.4. Binding of [^{125}I]T3 to rat liver nuclei in the absence (●) or presence of oleic acid (■) and an excess of non-radioactive T3 (▲), respectively (top panel). Dissociation of bound [^{125}I]T3 occurred by the addition at 120 min of either an excess of non-radioactive T3 (△), or oleic acid (□), or a combination of both agents (○) (top and bottom panels).

decreased the inhibition of nuclear T3 binding by oleic acid, both when added together with oleic acid and when added after oleic acid; it did so in a dose-dependent manner (not shown). It is observed that 0.5 mg/ml BSA is capable of almost complete neutralisation of the inhibitory effect of

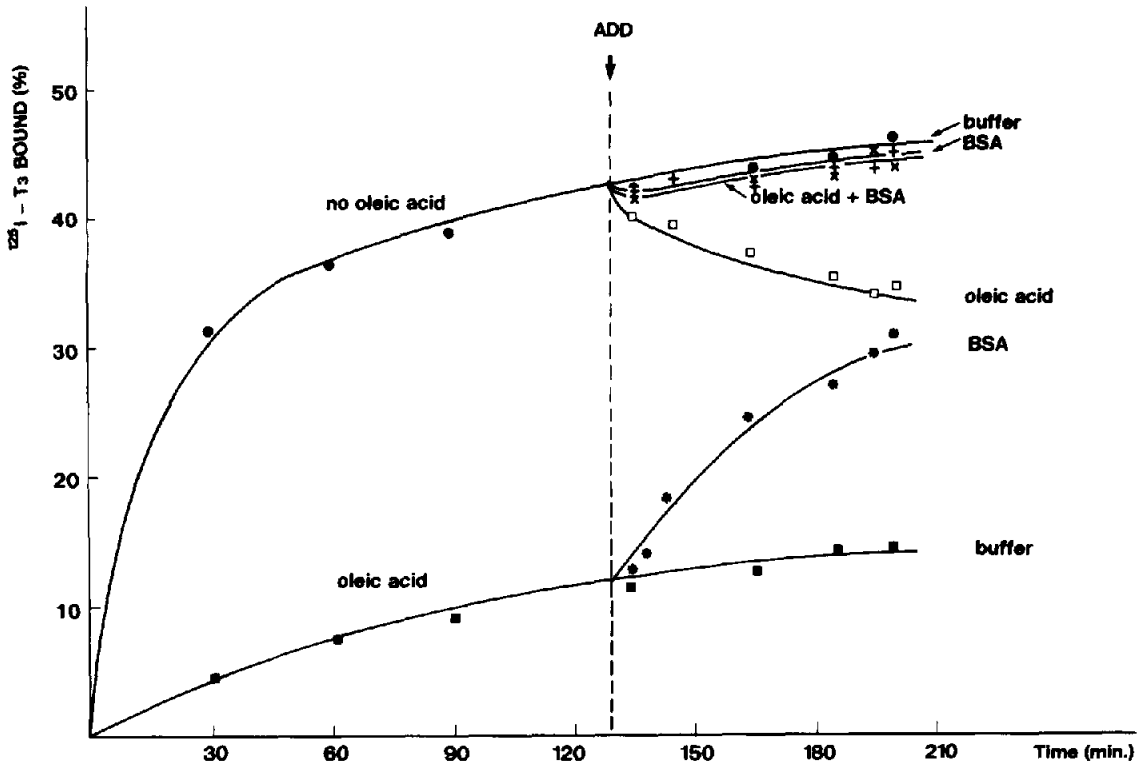

Fig.5. Binding of [^{125}I]T3 to nuclei in the absence (●) or presence of 0.33×10^{-4} M oleic acid (■). The dissociation of bound [^{125}I]T3 by the delayed addition of oleic acid (□) was abolished by the simultaneous addition of 0.5 mg/ml BSA (+); delayed addition of BSA (★) to incubation mixtures already containing oleic acid also diminished the inhibitory effect on nuclear T3 binding.

0.33×10^{-4} M oleic acid. From these data we calculated that one molecule of BSA was required to neutralize 4.4 molecules of oleic acid.

4. DISCUSSION

The studies reported here attempt to clarify the mechanism by which unsaturated fatty acids inhibit nuclear T3 binding. Oleic acid had no effect on equilibrium dialysis of T3. Moreover, the diffusion rate constant of [^{125}I]T3 as calculated from symmetric dialysis was not affected by oleic acid. Therefore, inhibition of nuclear T3 binding by oleic acid cannot be explained by decreased availability of T3 for receptor binding that would occur if T3 interacted directly with the fatty acid. Our experiments on inhibition of nuclear [^{125}I]T3 binding by the simultaneous addition of oleic acid and an excess of T3 (fig.4) also indicate the absence of a direct interaction between the hormone itself and the fatty acid.

As is evident from Scatchard analysis, the inhibition of nuclear T3 binding by oleic acid was caused by a marked reduction in apparent K_a, in agreement with our previous study [3]. The inhibition constant of oleic acid was 10^4 times greater than the inhibition constant of T3. It was in the same order of magnitude as the K_i of unsaturated fatty acids that inhibit the binding of angiotensin to their receptors in adrenal glomerulosa cells [1]. The Lineweaver-Burk plots demonstrate a strict competitive inhibition of nuclear T3 binding by oleic acid.

This is in contrast to the non-competitive inhibition by unsaturated fatty acids on the binding of tamoxifen to anti-oestrogen-binding sites as reported by Hwang [2]. The reason for this discrepancy is unclear. Lineweaver-Burk analysis of Hwang's data would possibly give more informa-

tion about the nature of inhibition by fatty acids in his study. The competitive nature of the binding inhibition by oleic acid suggests that the fatty acid competes directly with T3 for the same binding site on the nuclear receptor. However, competitive inhibition is also compatible with an allosteric hindrance of the ligand-receptor binding [8]. Because the inhibitory effect of unsaturated fatty acids is not limited to the T3 receptor, we consider an allosteric hindrance of nuclear T3 binding by oleic acid as the most likely explanation.

Studies with delayed addition of oleic acid and/or BSA indicated the reversible nature of the inhibitory effect on nuclear T3 binding. The affinity of oleic acid for the nuclear T3 receptor was obviously smaller than for BSA, and the calculated number of 4.4 oleic acid molecules bound per albumin molecule did not exceed the known number of 7 binding sites with a high to moderate affinity for long chain fatty acids on albumin [9]. The observed decrease of inhibition of nuclear T3 binding by oleic acid could therefore be totally accounted for by the binding of the fatty acid to BSA.

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