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8. We made the best possible print from each film. However, the appearance of the prints differs from that of the films in two cases: (i) the image of the underexposed radiograph is enhanced by printing on high-contrast paper; and (ii) the background fog of the autoradiograph is higher than that of the optimum exposure, and this does not show on the print.
9. Industrial x-ray film was used only because it was readily available in the laboratory. The speed of the various types of Kodak x-ray film decreases in the order AA, M, and R; the time required to make an autoradiograph increases by about a factor of 2 as the speed of the receiver film decreases.
10. The agitation problem does not preclude automation of the process because a similar technique can be incorporated in the processing machine. Also, we have not tried gaseous burst agitation.

11. I thank G. Rao and A. Brill of Vanderbilt University Hospital for supplying the films and participating in the evaluation; R. Wagner, G. Barnes, E. Kerkes, and R. O'Dell for discussions and help; and D. Speich for the photography.

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Fasting Decreases Triiodothyronine Receptor Capacity

Abstract. *Fasting decreases the ratio of hepatic nuclear to serum triiodothyronine (T_3) by diminishing the binding capacity of nuclear T_3 receptors. In combination with the lower serum T_3 concentration caused by fasting, the decrease in receptor content results in a marked decrease in nuclear T_3-receptor complexes. The changes in T_3 receptor content and circulating T_3 in fasted animals appear to be independent synergistic adaptations for caloric conservation in the fasted state. Unlike changes in hormonal level, the modification of nuclear receptor content provides a mechanism that may protect cells with a low caloric reserve independently of the metabolic status of the whole animal.*

Fasting decreases the serum concentration of 3,5,3′-triiodothyronine (T_3), concomitantly increasing the concentration of 3,3′5′-triiodothyronine (reverse T_3) (*1*). The latter has little or no calorigenic activity (*2*). The reduction in serum T_3 is probably a mechanism for caloric conservation in the fasted state. However it is the T_3-receptor complex rather than T_3 itself that is required for a hormonal effect, and a decreased concentration of serum T_3 does not necessarily imply fewer T_3-receptor complexes. The number of such complexes is determined both by the concentration of T_3 available for binding and the number and association constants of T_3 receptors. Several hormones diminish the concentrations of their own receptors (down regulation), thus tending to neutralize the biological effects of sustained changes in their concentration (*3*). This may also be true of T_3. Down regulation of the pituitary nuclear T_3 receptor by exposure to T_3 has recently been reported (*4*). Of several putative T_3 receptors (and sites of action), the nuclear receptor is the best characterized and thus far is the only receptor for which a clear correlation between occupancy and biological action has been established (*5–7*). The purpose of the present study was to determine whether fasting affects the nuclear T_3 receptor. We report that fasting decreases the ratio of hepatic nuclear T_3 to serum T_3 by decreasing the nuclear content of T_3 receptors.

Female Sprague-Dawley rats weighing between 150 and 250 g were used. Rats of similar weight were paired and placed in individual cages with free access to water. Food was withheld from one of the pair. For studies of nuclear T_3 uptake in vivo, [^{125}I]T_3 in dilute rat serum was injected into the femoral artery. This procedure and subsequent exsanguination through the abdominal aorta were carried out under light ether anesthesia. The rats were exsanguinated 30 minutes after injection. This is sufficient time for T_3 to equilibrate with rat nuclei (*8*). The livers were removed and chilled on ice. All subsequent procedures were carried out at 0° to 4°C. A weighed portion of liver, dissected free of fibrous tissue, was homogenized in ten volumes of 0.25*M* sucrose in 20 m*M* tris buffer, *p*H 7.85, containing 1 m*M* $MgCl_2$. The homogenate was centrifuged for 10 minutes at 600*g* and the pellet resuspended by gentle homogenization with 0.5 percent Triton X-100 in the sucrose solution. The Triton treatment was repeated once more and the final pellet was washed and resuspended in sucrose. Portions of the nuclear suspension were taken to determine radioactivity as well as DNA (*9*). The portion taken for determination of radioactive content was counted in an automatic well-type gamma scintillation counter without further preparation. For determination of DNA, a separate portion of the nuclei was washed three times in tris buffer to remove sucrose. The DNA was measured by the method of Ceriotti (*10*). The solubilized T_3 receptor was extracted from nuclei by the method of Samuels *et al.* (*11*). Total serum T_3 was determined by solid phase immunoassay (*12*) of unprecipitated serum. Serum portions were precipitated with 10 percent trichloroacetic acid before counting to separate [^{125}I]T_3 from [^{125}I]iodide.

Triiodothyronine binding studies were carried out in vitro by incubating the nuclear extract with tracer quantities of [^{125}I]T_3 and separating bound from free T_3 with dextran-coated charcoal (*13*). The charcoal was prepared by mixing 300 mg of Norit-A and 30 mg of dextran-15 in 100 ml of 0.4*M* KCl in sucrose buffer, *p*H 7.85. A 0.5-ml portion of extract containing [^{125}I]T_3 was added to 1 ml of dextran-charcoal in an ice bath, vortexed, and allowed to stand for 15 minutes. The charcoal was separated by centrifugation and the decanted supernatant and charcoal were counted in a gamma scintillation counter to determine bound and free [^{125}I]T_3.

Rats fasted for 5 days remained active and appeared to be in good condition. The results of the experiments in vivo are summarized in Table 1. With fasting, serum T_3 concentrations decreased on the average by about 50 percent. Liver weights decreased with a relative conservation of DNA so that the DNA/liver weight ratio increased proportionately. The ratio of hepatic nuclear to serum

Table 1. Effects of a 5-day fast on the nuclear/serum ^{125}I ratio after injection of [^{125}I]triiodothyronine.

Measure	*N*	Control rats	Fasted rats	*P*†
Weight change (g)	6	+21.7 ± 6.6*	−75.8 ± 17.1	<.001
Serum T_3 (ng/dl)	5	48.2 ± 10.0	24.4 ± 13.7	<.025
Liver weight (g)	6	9.8 ± 1.3	5.5 ± 0.9	<.01
Liver DNA (mg/g)	6	0.97 ± 0.10	2.0 ± 0.28	<.001
Nuclear/serum ^{125}I (cpm/μg DNA)/(cpm/μl serum)	6	0.94 ± 0.11	0.65 ± 0.18	<.005

*Mean ± S.D. †Paired *t*-test.

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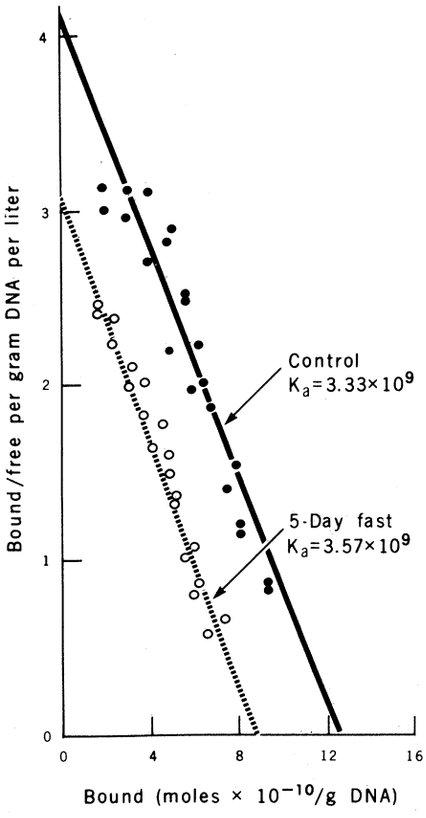

Fig. 1. Scatchard analysis of the binding of $[^{125}I]T_3$ to extracts of nuclei from fasted and control animals. Incubation was at 0°C.

$[^{125}I]T_3$ as measured by (counts per minute per microgram of DNA)/(counts per minute per microliter of serum) decreased significantly in the fasted animals in six paired experiments. Virtually all of the nuclear T_3 binding was to saturable sites. Injection of 40 μg of nonisotopic T_3 with $[^{125}I]T_3$ decreased the nuclear/serum $[^{125}I]T_3$ ratio to less than 0.02. The decreased nuclear/serum ^{125}I ratios in fasted animals imply a decrease in nuclear T_3 content that is more than proportional to the decrease of serum T_3.

The diminished uptake of $[^{125}I]T_3$ by nuclei in vivo could be due to factors other than modification of the T_3 receptor itself. We undertook further studies to determine whether fasting diminished the capacity or affinity of the hepatic nuclear receptors. Nuclei were extracted with a constant volume of KCl buffer per gram of initial tissue (method A). The ability of the nuclear extract to bind T_3 was measured as the ratio of bound to free $[^{125}I]T_3$ per gram of DNA per liter. In ten paired experiments with animals fasted 4 or 5 days, the value of this measure in controls was 2.18 ± 0.52 (standard deviation) and in fasted rats 1.29 ± 0.50 ($P < .001$, paired t-test). In an additional five experiments, the DNA content of the nuclear preparations was determined before extraction. Control nuclei were extracted as above, but for nuclei from fasted rats the extraction volume was increased in proportion to the greater DNA content of the nuclear preparation from these animals (method B). The results were similar to those obtained with method A. The ratio of bound to free $[^{125}I]T_3$ per gram of DNA per liter was 1.94 ± 0.29 in control extracts and 1.32 ± 0.27 ($P < .001$) in extracts from fasted rats (*14*). Saturation studies were carried out on extracts from paired control and 5-day-fasted rats. The content of endogenous T_3 was estimated from the serum T_3 concentration and the ratio of nuclear T_3 to serum T_3 determined in the preceding nuclear uptake studies in vivo. For the Scatchard analysis, slopes and intercepts were calculated by the least-squares linear regression method. The Scatchard analysis (Fig. 1) shows that fasting decreases the T_3 receptor capacity (abscissa intercept). Parallel slopes indicate that fasting had little or no effect on the association constant (K_a) of the T_3 receptor.

In two additional experiments, some decrease of K_a was found with fasting but the effect on receptor capacity predominated. The K_a of the control extract was $3.3 \times 10^9\ M^{-1}$, which is of the same order of magnitude as the K_a of 6.3×10^9 obtained by Samuels *et al.* (*11*) with incubation of solubilized nuclear receptors at 0°C. The receptor capacity of $12.2 \times 10^{-10}\ M$ per gram of DNA is equivalent to 0.78 ng per milligram of DNA. This is in good agreement with the binding capacity of 0.6 ng/mg DNA in hepatic nuclei reported by Surks *et al.* (*15*).

As an additional approach to the question of whether the number or the affinity of nuclear receptors was decreased by fasting, a timed study of $[^{125}I]T_3$ uptake and release was carried out with the solubilized receptor. Solubilized receptor was prepared from control nuclei and fasted nuclei diluted to the same DNA content as the controls. In Fig. 2, A and B, binding is represented as bound/total rather than bound/free so that the release rate can be shown. The decreased equilibrium bound/total ratio of the receptor preparation from the fasted animals was not associated with any slowing of the approach to the equilibrium level of binding. With the addition of a saturating concentration of nonisotopic T_3 at 24 hours, the release rate was similar for both preparations, with a half-time of 12.5 hours. Thus the decreased T_3 binding in the extract from fasted rats is due neither to a decreased rate at which

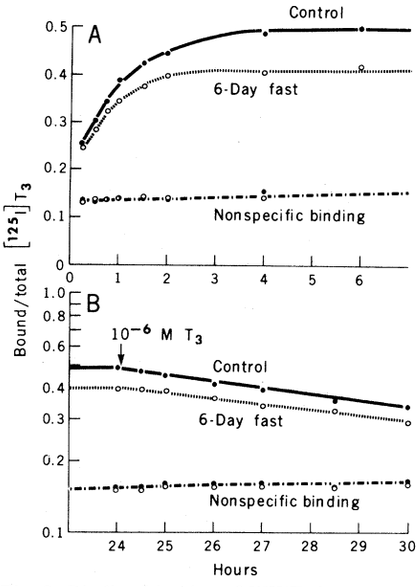

Fig. 2. Binding kinetics of $[^{125}I]T_3$ in extracts of nuclei from 6-day-fasted and control rats. The nuclei from fasted rats were diluted to the same DNA content as control nuclei before extraction. Nonspecific binding was determined by incubation in $10^{-6}M$ T_3. (A) The rate of binding was followed by measuring bound/total $[^{125}I]T_3$ at time intervals after the addition of $[^{125}I]T_3$. (B) At 24 hours a saturating concentration ($10^{-6}M$) of T_3 was added to determine the rate of dissociation of T_3-receptor complexes.

available binding sites are occupied nor to a more rapid release of bound T_3. These results are consistent with the saturation studies which show that fasting decreases the capacity rather than the K_a of the nuclear T_3 receptors.

Decreased T_3 concentration per se does not diminish the hepatic nuclear T_3 receptor content (*16*). Therefore the decreased serum T_3 and nuclear T_3 receptor capacities are probably independent but synergistic effects of fasting. It is of particular interest that the nuclear T_3 receptor can change independently of T_3 concentration (*17*). Although other hormonal factors are not excluded, it is likely that the cell is able to modify the nuclear T_3 receptor content in response to its own metabolic status, which infers the possibility of individual target cell acceptance or rejection of the hormonal directive (*18*). The decrease in the serum concentration of T_3 in response to starvation is probably an important adaptation to prolong survival of the organism. Decreased nuclear receptor content may be an equally important mechanism to protect individual cells when their caloric reserve is low (*19*).

GEORGE C. SCHUSSLER
JOSEPH ORLANDO
Mount Sinai School of Medicine, New York 10029

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19. While this work was in press, decreased nuclear T_3 receptor content during fasting was reported by K. D. Burman, Y. Lukes, F. D. Wright, and L. Wartofsky [*Endocrinology* **101**, 1331 (1977)], and by L. J. DeGroot, A. H. Coleoni, P.A. Rue, H. Seo, E. Martino, and S. Refetoff [*Biochem. Biophys. Res. Commun.* **79**, 166 (1977)].

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Covalent Immunoglobulin Assembly in vitro: Reactivity of Light Chain Covalent Dimers (L_2) and Blocked Light Chain Monomers

Abstract. *Covalent light chain dimers (L_2) and cysteine-blocked L chain monomers readily react with partially reduced heavy (H) chains. A rapid disappearance of these blocked L chain species is followed by the appearance of covalent intermediates—HL, H_2, and H_2L—leading to fully assembled H_2L_2. The mechanism of initial disulfide bond formation between heavy and light chains is disulfide interchange.*

In a study of the factors controlling antibody assembly, we have investigated in vitro the covalent assembly of a human IgG1κ immunoglobulin (Fro) (*1*). The quaternary structure of proteins of this immunoglobulin class is maintained by strong noncovalent forces as well as by four covalent interchain disulfide bonds. Two of these link the heavy (H) chains to each other, and two bridge heavy to light (L) chains, utilizing the carboxy terminal light chain residues and residue 220 in each heavy chain (*2*).

The interchain disulfides can be reduced without detectable reduction of any of the intrachain bonds (*3, 4*). Our experimental procedure, accordingly, entails selective (partial) reduction of the four interchain disulfide bonds, followed in certain experiments by reoxidation without prior disruption of the noncovalently associated tetramer (*3*) and in others by reoxidation after the H and L chains are separated and then recombined in varying molar ratios (*4*). The last type of experiment permits study of the assembly and reoxidation processes when L chains are modified, or present in excess, as is often the case both in normal lymphoid cells (*5, 6*) and in mouse myeloma tumors or cloned cell lines (*7*).

Methods have been devised for the quantitative determination of assembly intermediates during reduction as well as reoxidation (*3, 8*), so that not only initial and final states, but the kinetics of conversion from one to the other can be analyzed. Moreover, a general theory has been formulated to compare assembly in vitro with reported intracellular pathways and kinetics of assembly (*9*).

Among the key experimental findings with the IgG1κ studied are (i) that the rates and pathways of covalent assembly are not significantly affected by whether or not the H and L chains are separated from one another prior to reoxidation at neutral *p*H, and (ii) that large molar excesses of L over H chains do not markedly affect the pathways compared to equimolar reoxidations, although excesses diminish the rate of covalent H_2L_2 assembly. At the same time, excess L chains retard the formation of insoluble H chain aggregates in vitro, and this suggests a comparable role for such excesses in vivo (*4*).

The theoretical analysis revealed that the reoxidation process is not random; the four interchain disulfide bonds form with unequal probability throughout the course of the reaction. Initially, HL bonds form about twice as rapidly as HH bonds, but the formation of the second HL bond in the molecule is not favored over HH bond formation. In other words, the existence of one HL bond in a noncovalently assembled tetramer slows the rate at which the second forms.

Preliminary results have also established that covalent L_2 dimers rapidly react with reduced H chains or H_2 dimers and generate intermediates that assemble normally into H_2L_2 tetramers, a finding unanticipated by any previous studies on immunoglobulins (*10, 11*). Our report further documents this result and demonstrates also the reaction of partially reduced H chains with a stable L chain monomer whose carboxy terminal cysteine residue is in mixed disulfide linkage with the amino acid L-cysteine (*12*).

These L chain forms are derived from the urinary Bence Jones protein. Chromatography on Sephadex G-100 yields two fractions, a covalent L chain dimer and a stable L chain monomer. These forms are not interconvertible, and neither contains detectable free sulfhydryl (*12*). Procedures for preparation and purification of the plasma monoclonal protein (Fro) and of the partially reduced H chains have been published (*3, 4*), as have all of the other analytical procedures employed in this study (*3, 4, 11*). For recombination experiments, reduced H chains and unreduced dimeric or

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