raising neuronal serotonin levels, tryptophan increases the amount of serotonin released by a given dose of fenfluramine.) Rats were treated with tryptophan (100 mg per kilogram of body weight) or its diluent 30 minutes before receiving a dose of fenfluramine that would not in itself modify food consumption (1 mg per kilogram) (Fig. 2). Those receiving both the amino acid and fenfluramine consumed no less food during the next 4 hours than control animals (5.25 as compared to 5.20 g); however, they ate significantly more protein [as the percentage of total calories (29 ± 3 as compared to 21 ± 3, $P < .05$)]. Our data thus support a relation between serotoninergic transmission and protein consumption but suggest that, under the conditions of our experiment, this relation differs from that proposed earlier (*3*)—that is, serotonin release by anorectic drugs preserves, and somehow stimulates, protein intake. Additional support for the view that brain serotonin mediates fenfluramine's differential effects on protein and calorie consumption was obtained from studies with another anorectic drug, *d*-amphetamine (2.5 mg per kilogram of body weight, intraperitoneally), whose actions in the central nervous system are thought to depend on the release of catecholamines, not serotonin (*11*). Amphetamine doses that consistently reduced food consumption never altered the proportion of total calories consumed as protein.

These observations suggest the following: (i) Anorectic drugs can differentially affect protein consumption regardless of calorie consumption. Drugs that sustain protein intake while reducing the consumption of calories may have special value in the long-term management of obesity. (ii) Protein consumption apparently is facilitated by anorectic drugs that increase intrasynaptic serotonin, but is not spared by anorectic agents that act primarily at nonserotoninergic synapses. (iii) Different neuronal pathways thus underlie the mechanisms controlling the elective consumption of proteins and total calories (*12*).

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5. Both experimental diets contained (per kilogram): 150 g of corn oil, 22 g of vitamin mix (ICN Pharmaceuticals, Cleveland, Ohio), 40 g of Harper-Rogers Mineral Mix (Tekland Test Diets, Madison, Wis.), 35 g of agar, and 1000 ml of water. The 5 percent protein diet contained, in addition, 50 g of casein and 800 g of dextrin per kilogram; the 45 percent protein diet contained 450 g of casein and 400 g of dextrin per kilogram. Diets were put in individual feeding dishes; the locations of these pans within the cages were shifted daily.
6. The sucrose diets contained 23 percent casein, 5 percent corn oil, and agar, vitamin mix, and mineral salts as described in (*4*). The 24 percent sucrose diet contained 24 percent sucrose, 24 percent dextrin, and 24 percent dextrose; the 48 percent sucrose diet contained 48 percent sucrose, 12 percent dextrin, and 12 percent dextrose.
7. Fenfluramine was provided by the A. H. Robins Co., Richmond, Va.; fluoxetine was provided by the Eli Lilly Research Laboratories, Indianapolis, Ind.
8. In a typical experiment with 28-day-old rats, fenfluramine administration decreased consumption of the 5 percent casein diet from 3.1 to 0.5 g during this interval and increased consumption of the 45 percent casein diet from 3.0 to 4.0 g. Caloric intake thus fell from 29.0 to 21.3, whereas protein consumption rose from 1.50 to 1.82 g among rats receiving the drug.
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"Bound Water" in Barnacle Muscle as Indicated in Nuclear Magnetic Resonance Studies

In their report on transverse nuclear magnetic resonance (NMR) relaxation in barnacle muscle, Foster *et al.* (*1*) described useful experiments. However, we think that their conclusion that "one water molecule per thousand, which is briefly and irrotationally bound, will produce the observed relaxation properties" of muscle water is misleading and that there are problems with the approach used.

1) To explain the relaxation time T_2 of muscle water, Foster *et al.* assumed a two-fraction exchange model in which the "bound" water molecule is "irrotationally bound." This assumption is not supported by other experimental evidence. In fact, it has been shown that such a simplified assumption is incorrect for biological systems: on the basis of proton, deuteron, and oxygen-17 relaxation data, Koenig *et al.* (*2*) found that the two-fraction exchange model with irrotationally bound water failed to explain the relaxation times observed in protein solutions. Held *et al.* (*3*) studied the frequency dependence of the relaxation time T_1 in skeletal muscle, and their experimental results were also incompatible with the model of irrotationally bound water. Woessner and Snowden (*4*) found evidence for anisotropy of the rotational motion of bound water in agar solutions, which would not be obtained if the water molecules were irrotationally bound. The assumption of Foster *et al.* is therefore apparently incorrect.

2) It has been estimated from freezing experiments (*5*, *6*) that the fraction of bound water in skeletal muscle is of the order of 10 to 20 percent. Foster *et al.* reported that "this nonfreezing water [which is ascribed to bound water] is equivalent to about 0.07 g of water per gram of wet tissue." (Taking the dry weight of barnacle muscle as 30 percent, their figure is equivalent to 10 percent bound water.) They also reported that this water exchanges with D_2O. We think that their approach may not adequately take into account the contribution of this water to the relaxation of the major portion of the cell water.

3) If Foster *et al.* draw a distinction between their observed nonfreezing bound water and their theoretical irrotationally bound water, they are, in effect, proposing two kinds of bound water. Since they argued that the nonfreezing bound water exchanged with the rest of tissue water, the relaxation effect of the nonfreezing water cannot be ignored. According to figure 2 in (*1*), the nonfreezing water has a T_2 of about 100 μsec. For temperatures below 243°K, Belton *et al.* (*6*) gave an activation energy of 5.2 kcal/mole for the temperature dependence of T_2 of the unfrozen water in frog muscle. Using this activation energy, we extrapolate 100 μsec at −34°C to 878 μsec at 298°K. If there is rapid exchange between the nonfreezing bound water and the muscle water, as Foster *et al.* implied, the T_2 of the whole tissue water at 298°K would be approximately 8.8 msec. The relaxation effect of this nonfreezing water alone is more than enough

to account for the T_2 observed. Thus, the proposed irrotationally bound water is unnecessary. Furthermore, if the irrotationally bound water indeed exists and is different from the nonfreezing bound water, the correct treatment of the problem should involve a calculation of the exchange between three fractions of water molecules. Equation 1 of Foster *et al.* is not correct.

4) If one argues that the irrotationally bound water is part of the nonfreezing water, and the T_2 relaxation of the nonfreezing water at −34°C is dominated by roughly 1 percent irrotationally bound water so that the mobility of the other 99 percent is extremely great, there is still a serious discrepancy. Consider the relaxation time at −34°C from equation 1 of Foster *et al.*

$$T_{2\mathrm{obs}} = \frac{x}{P_\mathrm{b}} T_{2\mathrm{b}} + \tau_\mathrm{a} \qquad (1)$$

where P_b is the fraction of irrotationally bound water, τ_a is the average lifetime of nonfreezing water with large mobility, $T_{2\mathrm{b}}$ is the proton T_2 of irrotationally bound water, and $x = 1.0$ for proton water. Since the first term on the right side of equation 1 is larger than zero, $\tau_\mathrm{a} < T_{2\mathrm{obs}}$. At −34°C $T_{2\mathrm{obs}}$ is about 100 μsec, so τ_a must be smaller than 100 μsec at that temperature. But according to figure 3 in (*1*) the average lifetime of a water molecule *not* in the irrotationally bound phase is about 35 msec at room temperature. Since the nonfreezing water with large mobility is about 10 percent of the total tissue water, by detailed balance, τ_a must be approximately 3.5 msec. This means that τ_a increases with temperature. However, this result is opposite to the known temperature dependence of τ_a in liquid water and would imply a negative activation energy for water.

5) Finally, in figure 3 of Foster *et al.* the plotted data do not agree with their prediction. Regardless of the rate of exchange, their equation 1 should predict a straight line in the plot of $T_{2\mathrm{obs}}$ against x, while the data seem to show a large degree of curvature. The curvature probably results from a failure of equation 1 to include proton relaxation which is independent of the isotopic composition of the water, such as interaction between water protons and nonexchangeable macromolecular protons (*4*). In general, the T_2 of bound water can be calculated from

$$\frac{1}{T_{2\mathrm{bound}}} = \alpha + f_\mathrm{H}\beta + \frac{1}{L}(1 - f_\mathrm{H})\beta$$

$$= \alpha + \frac{\beta}{L} + \beta\left(1 - \frac{1}{L}\right) f_\mathrm{H}$$

where α is the relaxation effect from macromolecular protons, β is the relaxation from intramolecular dipolar interaction, L is the ratio of second moments between proton and deuteron, and f_H is the isotopic fraction of protons in tissue water. Our β here is equivalent to the inverse of $T_{2\mathrm{b}}$ in equation 1 of (*1*). A comparison of the equation given above with that of Foster *et al.* indicates that their x should be defined as

$$x = \frac{1}{a + bf_\mathrm{H}}$$

where $a = \alpha/\beta + 1/L$ and $b = 1 - 1/L$. Foster *et al.* defined x as $1/f_\mathrm{H}$. If a is significantly large in comparison with bf_H, it is not surprising that the graph of $T_{2\mathrm{obs}}$ against x deviates from a straight line.

In conclusion, we find that there are serious contradictions between the results of the analysis of Foster *et al.* and the experimental observations. Their basic assumption was unsound, and their conclusion as stated in their abstract was misleading. A casual reader may be misled to believe that only 0.1 percent of muscle water is bound. It was not clear in the report by Foster *et al.* how they differentiate between the observed nonfreezing bound water and the theoretical irrotationally bound water. We find that, in all cases, the approach adopted by Foster *et al.* would lead to results contradicting the observations on the nonfreezing water, the relaxation effect of which was neglected in their treatment.

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7 March 1977

The current situation with respect to the theory of nuclear magnetic resonance (NMR) phenomena in biological waters remains one of model building and hypothesis testing. Chang and Woessner (*1*) evidently believe that our recent research report on certain NMR phenomena of water in muscle (*2*) advocates too strongly a model with which they do not fully agree, mainly because of the narrow range of phenomena they see the model to encompass.

The model (*2, 3*) of intracellular water in question is that of relatively mobile water exchanging with a small number of bound water molecules (less than one per thousand); the bound molecules are held in a fixed orientation (irrotationally) for an average binding time, τ_b, of several microseconds, such that τ_b is identically the NMR correlation time in the bound state; the binding time or lifetime in the bound state is sufficiently long that fast exchange [in the NMR sense (*4*)] between the two sorts of molecules does not occur. We refer to this as the model of irrotationally bound water (IBW) with binding-time effects. In the IBW model there are only two free parameters: the number of bound molecules and the lifetime in the bound state. The experiments we reported (*2*) were novel in that both parameters could be determined. For consistency with NMR theories of motional narrowing, the binding time is required to be (*3*) [and is found to be (*2, 3, 5*)] on the order of several microseconds. The IBW model also predicts the value of the applied radio-frequency field at which a dispersion of the rotating-frame spin lattice relaxation time $T_{1\rho}$ occurs (*3*), as discussed further below. The significance of this model is that if it is correct, then NMR relaxation experiments tell very little about the mobility of most of the water molecules in muscle tissue, but reflect only the influence of a very few binding sites.

The two-fraction fast exchange (TFE) model, which has routinely been applied to water in biological systems (*6, 7*), may also have as few as two parameters, but one (say the bound fraction) must be chosen before the other (the mobility of the bound state) can be determined. In points 2, 3, and 4 of their comment, Chang and Woessner assume that the nonfreezing water, reported by others (*6, 7*) as well as in (*2*), is in some way the bound water fraction required by any TFE model and criticize the IBW model for not being in harmony with their interpretations. Certainly we studied the nonfreezing water, showed that it exchanges with D_2O, and thereby showed that it is not identical to the observed proton fraction with a T_2 in the millisecond range (*2*); thus bounds were placed on some commonly held notions of bound water. But this does not require us to devise a model which at once explains the NMR properties of unfrozen tissue and any

changes in these properties on freezing. We do not know how the NMR relaxation data in frozen tissue relate to NMR relaxation in unfrozen tissue; we do not know the mechanism of freezing or the alterations in structure and segregation caused by freezing. Thus, we feel neither competent nor obliged to respond to points 2, 3, and 4 above.

In their fifth criticism, Chang and Woessner correctly point out (i) that our model neglected the effect of dipolar interaction of the protons in the irrotationally bound water molecules with the protons in the macromolecular structure (*8*), and (ii) that inclusion of this effect might bring the model into better agreement with the data in the isotope dilution experiment (*2*). This has also been pointed out to us by Edzes and Samulski (*5*), who included this intermolecular contribution to T_2 for water molecules in the bound state in fitting our proton T_2 data for barnacle muscle, extracted its value, and found that it almost dominates the dependence on deuterium concentration in a manner consistent with rapid exchange. They pointed out that it is only the closeness of the deuterium and proton T_2's which requires the introduction of the effects of τ_b and allows determination of the free parameters in the IBW model. Inclusion of the intermolecular contribution does not disturb the validity of the "incipient motional narrowing" consistency check (*3*) referred to above. Edzes and Samulski reworked the barnacle T_2 data because they were able to derive, from cross-relaxation effects in the relaxation time T_1 (albeit of water in chicken muscle), an independent estimate of the intermolecular contribution to T_2 in the bound state; the latter agrees very well with that found from the isotope dilution measurements in barnacle muscle and suggests that the model they (and Chang and Woessner) propose for isotopic dilution is valid. The cross-relaxation effects also require (*5*) that water molecules be bound for times τ_b greater than a Larmor period (that is, $\tau_b \gtrsim 10^{-8}$ second); the parameters obtained from barnacle muscle (*2*, *3*, *5*) are consistent with this requirement ($\tau_b \sim 10^{-5}$ second). Inclusion of intermolecular contributions to T_2 for the protons of water molecules in the bound state does not therefore vitiate the IBW model, but rather sustains it.

It was pointed out (*1*–*3*) that the IBW model does not hold for protein solutions (*9*) and agar gels (*10*). We believe that these two systems may be sufficiently different from muscle tissue that the same theoretical model should not be required to explain the NMR properties of all of them. The rigid substrate (rigid for, say, tens of microseconds) required for the IBW model may be present in muscle and not in agar gels or protein solutions; it is, in fact, the purpose of the NMR experiments to ascertain these things. With regard to the T_1 dispersion data of Held *et al.* (*11*), we find support rather than contradiction of the model in question. As far as $T_{1\rho}$ and T_1 dispersion effects are concerned, the muscle systems are, according to the model, effectively in the exchange rate–limited relaxation regime (*3*, *12*) where dispersion reflects a local field or "root interaction strength" rather than a correlation time. The dispersion observed by Held *et al.* occurs as predicted (*3*) and as observed for frog (*13*) and mouse (*14*) muscle; further, the recent observation by Fung (*15*) that the proton and deuteron dispersion frequencies are not equal (as a fast-exchange model with a single motional process would require) but differ by a factor of ~ 3 is in agreement with this idea, as is the absolute value of the deuteron dispersion frequency (*16*).

The IBW model of one water molecule per thousand, briefly and irrotationally bound, can indeed account for a great many of the NMR properties of water in muscle, namely (i) the small value of T_2 generally observed for intracellular water; (ii) the dependence of the proton T_2 on isotopic composition in barnacle muscle, as extended by Chang and Woessner (*1*) and by Edzes and Samulski (*5*); (iii) the ratio of the deuterium transverse relaxation time to that of the protons in the same system; (iv) the dispersion of the rotating-frame relaxation times in mouse and frog muscle; and (v) the dispersion of both proton and deuteron spin lattice relaxation times in frog and mouse muscle. In addition, T_1 cross-relaxation effects in the intracellular water of chicken muscle (*5*) are consistent with the model as well (*17*).

In conclusion, we believe that the nonfreezing water which is often described as bound water (*6*, *7*) is not immediately relevant to the construction of an NMR model for nonfrozen tissue; the model of a small fraction of water, irrotationally bound and exchanging at a fast to intermediate rate ($\sim 10^5$ sec^{-1}), is consistent with a rather large number of phenomena observed in various muscle systems; there are no serious contradictions between the results of the analysis given (*2*, *3*) (as amended to include intermolecular effects) and the experimental observations originally presented.

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2 September 1977

Rate-Dependency Hypothesis

In their recent article "Mathematics underlying the rate-dependency hypothesis," Gonzalez and Byrd (*1*) make precise and explicit some facets of rate dependency that had been merely implicitly understood. They are concerned with ways of presenting behavioral results when the independent variable is rate of responding and rates have been recorded in the presence and absence of a drug.

1) By using the word hypothesis they emphasize that the relation between rate