tionality, as well as the aliphatic polyfunctional acids. The increase in concentration (with the period of oxidation) of the aromatic acids, especially the di and tricarboxylics, is further evidence for carbon–carbon bond cleavage and indicates the presence of some aromatic nuclei within the organic polymer (kerogen). The drastic increase of the dicarboxylic aliphatic acids with the duration of oxidation lends support to earlier conclusions regarding the aliphatic nature of the Green River Formation kerogen. The range (C_4–C_{25}) and smooth distribution of the dicarboxylic acids suggest a random aliphatic cross-linked polymer matrix. Further experiments to elucidate the structural attachments, rate of oxidation of certain structure types and reaction product and by-product distributions to be expected from this kind of treatment are in progress.

We thank Mrs Ellen Scott for technical assistance and the US National Aeronautics and Space Administration for support.

Received October 22, 1968; revised January 20, 1969.

[1] Eglinton, G., Scott, P. M., Belsky, T., Burlingame, A. L., Richter, W., and Calvin, M., in *Advances in Organic Geochemistry 1964* (edit. by Hobson, G. D., and Louis, M. C.), 41 (Pergamon, Oxford, 1966).
[2] Burlingame, A. L., Haug, P., Belsky, T., and Calvin, M., *Proc. US Nat. Acad. Sci.*, **54**, 1406 (1965).
[3] Hills, J., Whitehead, E., Anders, D., Cummins, J., and Robinson, W., *Chem. Commun.*, 752 (1966).
[4] Murphy, M. T. J., McCormick, A., and Eglinton, G., *Science*, **157**, 1040 (1967).
[5] Abelson, P. H., and Parker, P. L., *Carnegie Inst. Wash. Year Book*, **61**, 181 (1962).
[6] Lawlor, D. L., and Robinson, W. E., *Div. Pet. Chem. Amer. Chem. Soc.*, Detroit Meeting (May 9, 1965).
[7] Eglinton, G., Douglas, A. G., Maxwell, J. R., Ramsay, J. N., and Ställberg-Stenhagen, S., *Science*, **153**, 1133 (1966).
[8] Ramsay, J. N., thesis, Univ. Glasgow (1966).
[9] Douglas, A. G., Douraghi-Zadeh, D., Eglinton, G., Maxwell, J. R., and Ramsay, J. N., in *Advances in Organic Geochemistry 1966* (edit. by Hobson, G. D., and Spears, G. C.) (Pergamon, Oxford, in the press).
[10] Haug, P., Schnoes, H. K., and Burlingame, A. L., *Science*, **158**, 772 (1967).
[11] Haug, P., Schnoes, H. K., and Burlingame, A. L., *Chem. Commun.*, 1130 (1967).
[12] Haug, P., Schnoes, H. K., and Burlingame, A. L., *Geochim. Cosmochim. Acta*, **32**, 358 (1968).
[13] Burlingame, A. L., and Simoneit, B. R., *Nature*, **218**, 252 (1968).
[14] Dinneen, G. U., Van Meter, R. A., Smith, J. R., Bailey, C. W., Cook, G. L., Allbright, C. S., and Ball, J. S., *Bureau Mines Bull.*, 593 (US Govt. Printing Office, 1961).
[15] Robinson, W. E., Heady, H., and Hubbard, A., *Ind. Eng. Chem.*, **45**, 788 (1953).
[16] Robinson, W. E., Cummins, J. J., and Stanfield, K., *Ind. Eng. Chem.*, **48**, 1134 (1956).
[17] Hoering, T. C., and Abelson, P. H., *Carnegie Inst. Wash. Year Book*, **64**, 218 (1965).
[18] Forsman, J. P., in *Organic Geochemistry* (edit. by Breger, I. A.) (Pergamon, New York, 1963).
[19] Burlingame, A. L., and Simoneit, B. R., *Science*, **160**, 531 (1968).
[20] Burlingame, A. L., Haug, P. A., Schnoes, H. K., and Simoneit, B. R., in *Advances in Organic Geochemistry 1968* (edit. by Schenck, P. A., and Havenaar, I.) (Vieweg/Pergamon, Braunschweig, Germany, 1969).
[21] Schulze, P., Simoneit, B. R., and Burlingame, A. L., *J. Mass Spec. and Ion Phys.*, **2**, 181 (1969).
[22] Burlingame, A. L., in *Advances in Mass Spectrometry*, **4** (edit. by Kendrick, E.), 15 (The Institute of Petroleum, London, 1968).
[23] Burlingame, A. L., and Smith, D. H., *Tetrahedron*, **24**, 5759 (1968).
[24] Ryhage, R., and Stenhagen, E., *Arkiv Kemi*, **23**, 167 (1964).
[25] Ryhage, R., and Stenhagen, E., *Arkiv Kemi*, **15**, 545 (1960).

Evidence for the Existence of a Minimum of Two Phases of Ordered Water in Skeletal Muscle

by
C. F. HAZLEWOOD
B. L. NICHOLS
Department of Pediatrics, and
Department of Physiology,
Baylor College of Medicine, and
Texas Children's Hospital,
Houston, Texas 77025

N. F. CHAMBERLAIN
Esso Research and Engineering Company
Baytown, Texas 77520

NMR spectra of muscle water suggest that it exists in at least two ordered phases, which can be distinguished by the widths of their NMR signals, by deuterium exchange and by vacuum drying. Heat denaturation of muscle protein lessens this ordering.

THERE is evidence that macromolecules such as proteins can exert a major influence on the properties of cellular water. Szent-Gyorgyi's suggestion[1] that water molecules surrounding proteins are in an ice-like state has support[2], and there is evidence that water molecules near biological macromolecules may be in a state different from pure water[3–10]. This seems, in particular, to apply to skeletal muscle[11–13] and to peripheral nerve[14,15].

We have used nuclear magnetic resonance (NMR) spectrometry to study the state of water in muscle tissue[16]. NMR is well suited to this task because the width of the signal produced by water hydrogens is dependent on the motional freedom of the water molecules[17]. As the mobility of the water molecules increases, the line width decreases.

We conclude that muscle cell water exists in at least two observably different degrees of order; water in muscle tissue produces two NMR signals, one of which is much broader than the other, and both of which are significantly broader than the signal produced by free water. We investigated the phenomena which are known to cause broadening of NMR signals, and the only one which appears to be significant in muscle is restriction of the motional freedom of water molecules. We attribute this restriction to specific attractive forces acting on the molecules.

Adsorption of the water molecules on less mobile molecules will restrict their motional freedom, as will physical interference with the motion of one sample molecule by another (increased bulk viscosity). This is the chief cause of broadening of NMR signals from petroleum oils. Further, separate solid or liquid phases in the sample generally broaden the NMR signal because of the magnetic inhomogeneities they produce. It is conceivable that very large molecules, such as protein molecules, could produce a similar line broadening of the water in muscle cells. Finally, inhomogeneities in the applied static magnetic field, or the presence of paramagnetic ions or free radicals, will broaden the signal. Each of these factors must be investigated or eliminated

in any determination of the physical state of water in muscle.

A Varian *A*-60-*A* high resolution NMR spectrometer was used to determine water spectra. Freshly excised skeletal muscle samples were taken from rats and mice, dissected free of visible fat, nerve and connective tissue and packed into sample tubes. The sample tubes were capped to prevent evaporation. All observations were made with sample tubes spinning and at temperatures between 32°–37° C. The only resonance signal observed was that produced by hydrogen nuclei in muscle water. Muscle water spectra obtained several minutes and hours apart were identical, and precluded the need to use various physiological salt solutions. In addition, several different muscles (gastrocnemius, extensor digitorum longus and cardiac) and tissues were studied and all were found to produce broad water signals.

The Varian *DA*-60 operated in the wide line mode was also used. The readily exchangeable muscle water was removed by exchange with D_2O. The sample was then observed in the wide line mode. Additional procedures involved vacuum desiccation of the muscle samples, following in general the techniques of Blears and Danyluk[7].

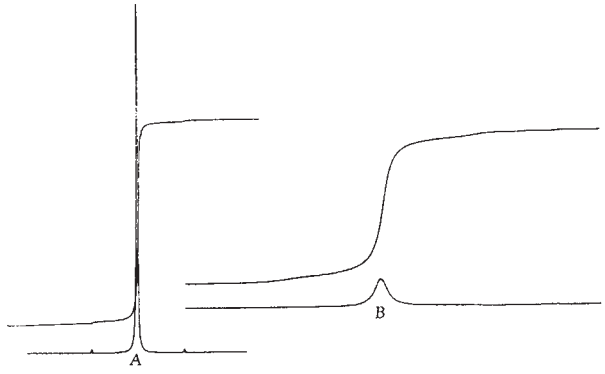

Fig. 1. High resolution NMR spectra and associated integrals of free water (*A*) and rat skeletal muscle water (*B*). Both spectra obtained using the same sample tube and machine settings. Sweep width = 1,000 Hz.

The NMR spectrum of distilled water and the associated integral are shown in Fig. 1*A*. The width at one-half amplitude[17] is 1 Hz (range of values 0·75–1·0 Hz). This narrow spectrum is characteristic of water molecules having the motional freedom of the liquid state. The water spectrum for the gastrocnemius muscle of a 32 day old rat, in the same conditions and machine settings, is much broader (15 Hz), and the spectrum integral is less than that for pure water. This has often been observed in mature rat and mouse skeletal muscle. The width at one-half amplitude varies from 12 to 17 Hz for skeletal muscle, and we attribute this range primarily to variations in sample packing. The spectra for pure water show that the line broadening due to inhomogeneities in the static magnetic field was 1 Hz or less.

The width and intensity of the muscle water spectrum (Fig. 1) suggest the presence of a minimum of two populations of water molecules subject to motional restriction. The broad signal indicates a large major phase of water molecules that are significantly ordered but have sufficient motional freedom to permit easy observation by high resolution NMR. The proportion of water in muscles of 32 day old rats is 80 per cent (ref. 16, and unpublished work of C. F. H. and B. L. N.) and if all the muscle water were visible to the NMR spectrometer the muscle water signal should be 80 per cent of the pure water signal. The integral of the muscle water spectrum is only 72 per cent of the pure water spectrum, however, suggesting the presence of a second or minor phase of water which produces a signal too broad to be detected in the high resolution scan. The minor phase, which is repeatedly indicated

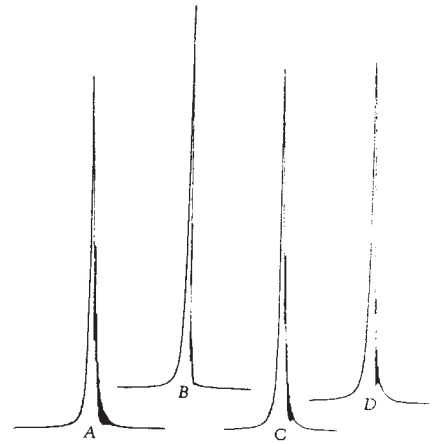

Fig. 2. High resolution NMR spectra of free water (*A*), Krebs Ringer (*B*), 2 per cent gelatine (*C*), and 10 per cent gelatine (*D*). Sweep width = 250 Hz.

in muscle water spectra, is variable and its exact magnitude must be statistically determined. Our studies indicate that it may be as large as 10 per cent of muscle water. The rest of this article is concerned with confirming the existence of at least a major and minor phase of ordered muscle water.

Evidence for the Existence of Ordered Water in the Major Phase

The broadening of the muscle water spectrum observed by high resolution NMR (Fig. 1*B*) could be due to any of the reasons discussed. We now present evidence that this broadening is due to the restriction of the motional freedom of the individual water molecules by their interaction with cellular macromolecules.

Concentrated solutions of potassium (up to 2 M) and sodium chloride (up to 2 per cent) were found not to affect the line width. Human plasma was also found to have a line width comparable with pure water. Krebs Ringer solution[18] also exhibited a narrow water line (Fig. 2*B*). Two and 10 per cent solutions of gelled gelatine did not broaden the line width of water (Fig. 2*C* and *D*). Increased viscosity or the presence of ions therefore need not cause the line broadening observed in Fig. 1*B*.

Agar can reduce the motional freedom of water molecules[19]. Two and 10 per cent agar solutions caused line broadening of 5·5 and 8·5 Hz. Narrowing of the water spectrum occurs after heating which is sufficient to liquefy the agar water solution (Fig. 3), and the broad line reappears on cooling. The line width of the warm agar

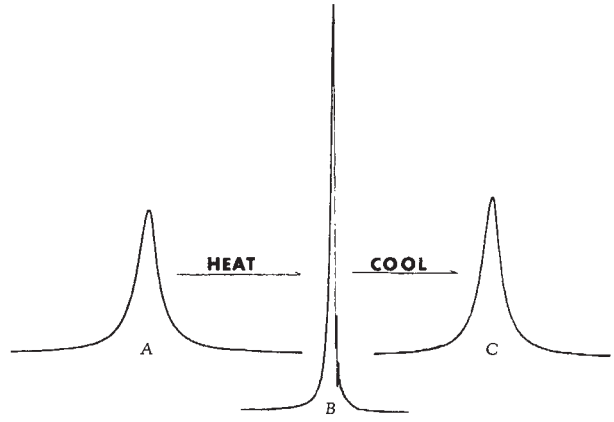

Fig. 3. High resolution NMR spectra of 2 per cent agar. The width at 1/2 amplitude is 5·5 Hz for the agar solution at room temperature (*A*). Moderate heating reduced the width at 1/2 amplitude to 1 Hz (*B*), and the width broadened to 5 Hz on cooling to room temperature (*C*). Sweep width = 250 Hz.

water solution (Fig. 3*B*) is identical to that of free water. Because the line narrowing occurred following heating (Fig. 3*B*) without change in chemical composition of the solution, we conclude that the mere physical presence of macromolecules is not responsible for the line broadening.

If the line broadening is due to specific interaction of water molecules with only the native cellular proteins[1,2,8–10,16] and other macromolecules, then a conformational change in macromolecular structure should release the orientated water. Fig. 4 shows spectra of rat skeletal muscle water before (*A*) and after (*B*) heat denaturation. The "normal" spectrum (Fig. 4*A*) shows a single broad water peak of 14·5 Hz, but after denaturation the line width narrows irreversibly to 3 Hz. Fig. 4*B* was recorded with the coagulated protein in the sample tube; therefore these denatured protein molecules do not significantly broaden the water line.

The results in Fig. 4 also rule out a significant contribution from paramagnetic impurities. In addition, the supernatant from a D_2O soaked muscle revealed a sharp peak with no line broadening, confirming the absence of water soluble paramagnetic impurities.

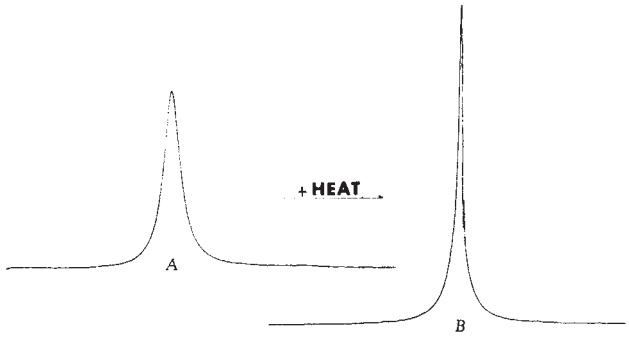

Fig. 4. High resolution NMR spectra of rat gastrocnemius muscle water before (*A*) and after (*B*) protein denaturation. The line width decreased from 14·5 Hz to 3 Hz. Sweep width = 500 Hz.

The remaining cause of NMR line broadening is specific interaction of the water molecules with native macromolecules in the muscle cell. This specific interaction restricts the motional freedom of the water molecules, and results in a more ordered system than is found in free water. The observation of only one broad water peak in the high resolution NMR spectra indicates that all the muscle water experiences, as an average, a significant restriction in its motional freedom. Muscle water limited to the extracellular phase should be free, and should be observed as a separate sharp peak unless exchange averaging of NMR signals is occurring. If restricted water molecules or their hydrogen nuclei exchange rapidly (on an NMR time scale) with free water molecules, the resulting NMR signal will be a single peak with a width which reflects the average environment of the hydrogen nuclei. We found that the addition of free water to a sample tube containing skeletal muscle results in a single water peak which is narrower than that for muscle water but still broader than that of pure water. When pure D_2O or D_2O Ringer is added to a similar sample, considerable narrowing of the remaining water signal occurs. This narrowing of the high resolution NMR signal to an intermediate width is consistent with exchange narrowing.

If a substantial volume of the muscle water were motionally free, but contained in a compartment which prevents rapid exchange with adsorbed water, it should be visible as a narrow line in the NMR spectrum. To check this notion, the following was done in the laboratory of Dr Paul A. Srere: (1) a spectrum was obtained on a skeletal muscle from a mouse (Fig. 5*A*); and (2) a spectrum of the same sample was obtained following the insertion of a capillary containing free water (Fig. 5*B*). The internal

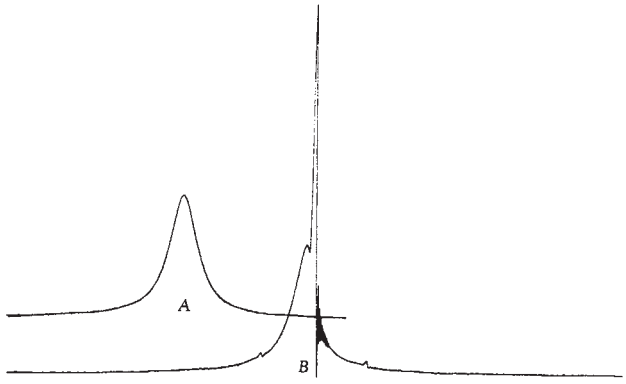

Fig. 5. High resolution NMR spectra of mouse skeletal muscle water. Spectrum *A* is of muscle alone and is 15 Hz wide. Spectrum *B* is the muscle with a capillary of free water placed in the bulk muscle phase. Sweep width = 500 Hz.

diameter of the capillary was 36 per cent that of the sample tube. The free water which is not permitted to exchange with the muscle mass is shown clearly in Fig. 5*B* as a narrow peak superimposed on the broad muscle water peak. Furthermore, the inhomogeneity and/or viscosity in the bulk muscle phase do not prevent the appearance of the sharp pure water signal (Fig. 5*B*).

Evidence for the Existence of Ordered Muscle Water in the Minor Phase

Protons of molecules in crystals, ice or other systems that have lost considerable motional freedom may be observed by wide-line NMR[7]. In the case of muscle tissue, major phase water produces such a strong NMR signal that it seriously interferes with observation of the minor phase. This strong signal is effectively removed by exchanging the major phase water hydrogens with deuterium.

We have completed a wide-line NMR study on skeletal muscle exchanged with D_2O for 24 h (Fig. 6*A*) and placed in a vacuum for 75 min (Fig. 6*B*). The broad signal component is attributed to macromolecules and the narrow component (approximately 1,000 Hz wide) is attributed to bound water in the minor phase. Fig. 7 is a wide-line NMR signal spectrum of skeletal muscle after 24 h of evacuation. The narrow signal component is still present, indicating that vacuum alone does not remove all the water. The motional restriction of the water molecules in the minor phase is comparable with that in polycrystalline ribonuclease which contains 19 per cent water or less.

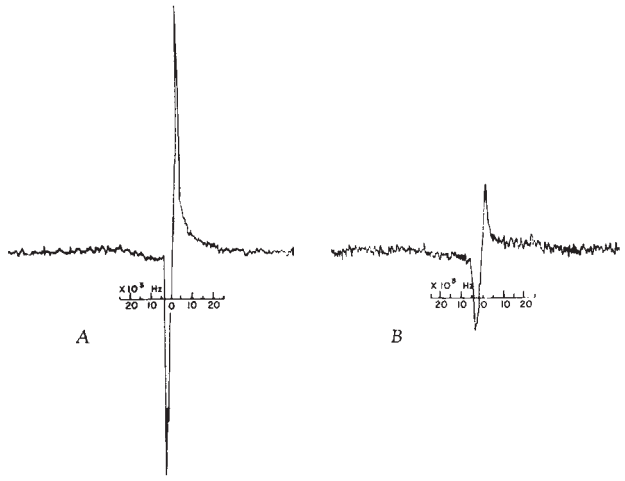

Fig. 6. Wide-line NMR spectra of rat skeletal muscle water following 24 h in D_2O (*A*) and 75 min in vacuum (*B*). The sharp signal is from the protons of the adsorbed water molecules, and this signal decreased following evacuation. The abscissae are calibrated in Hz.

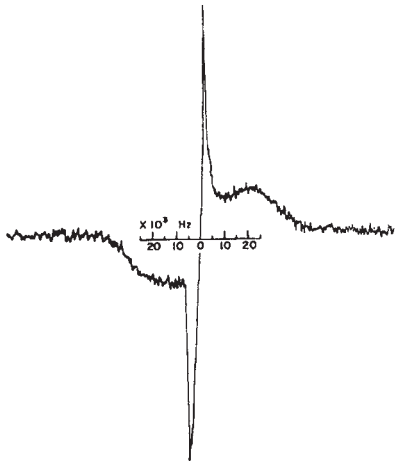

Fig. 7. Wide-line NMR spectrum of rat skeletal muscle following desiccation for 24 h in vacuum. The sharp signal is from the protons of the adsorbed water molecules and the broader signal is from the protein protons. The abscissa is calibrated in Hz.

We conclude from these studies that most of the skeletal muscle water experiences restricted motional freedom. At least two ordered phases of water have been observed and designated the major and minor phases. The major phase consists of water molecules that have lost considerable motional freedom relative to free water. The water hydrogens in the major phase exchange rapidly among themselves, and with those of free water added to the system. The minor phase contains water molecules that have less motional freedom than in the major phase but more than in solids.

It seems reasonable to speculate as follows: (1) the water molecules of the major phase experience a significant loss of translational motion and possibly some loss of rotational motion; and (2) the water molecules within the minor phase experience an even greater restriction in translational motion and a significant loss of some rotational motions.

Since we submitted our manuscript we have learned of work by Freeman Cope on the physical state of water in skeletal muscle. Cope, using a different approach, has concluded that skeletal muscle water is ordered and that two distinct fractions can be recognized. His work appeared in the *Biophysical Journal* in March 1969.

We thank Mr T. J. Denson and Mr T. Hines of Esso Research and Engineering for technical assistance, and Mr M. Gygli and Mr R. L. McGlothlin of St Lukes/Texas Children's Hospitals for photography. We also thank Dr S. S. Danyluk of the Argonne National Laboratory for his cooperation. This work was supported by grants from the Muscular Dystrophy Associations of America, Inc., and the US National Institutes of Health.

Received December 9, 1968; revised March 17, 1969.

[1] Szent-Gyorgyi, A., *Bioenergetics* (Academic Press, New York, 1957).
[2] Klotz, I. M., *Science*, **128**, 815 (1958).
[3] Berendsen, H. J. C., *J. Chem. Phys.*, **36**, 3297 (1962).
[4] Beeman, W. W., Geil, P., Shurman, M., and Malmon, A. G., *Acta Cryst.*, **10**, 818 (1957).
[5] Hearst, J. E., and Vinograd, J., *Proc. US Nat. Acad. Sci.*, **47**, 1005 (1961).
[6] Ritland, H. N., Kaesberg, P., and Beeman, W. W., *J. Chem. Phys.*, **18**, 1237 (1950).
[7] Blears, D. J., and Danyluk, S. S., *Biochim. Biophys. Acta*, **154**, 17 (1968).
[8] Ling, G. N., *J. Gen. Physiol.*, **43**, 149 (1960).
[9] Ling, G. N., *A Physical Theory of the Living State* (Blaisdell, New York, 1962).
[10] Ling, G. N., *Ann. NY Acad. Sci.*, **125**, 401 (1965).
[11] McLaughlin, S. G. H., and Hinke, J. A. M., *Canad. J. Physiol. Pharmacol.*, **44**, 837 (1966).
[12] Cope, F. W., *J. Gen. Physiol.*, **50**, 1353 (1967).
[13] Cope, F. W., *Bull. Math. Biophysics*, **29**, 583 (1967).
[14] Chapman, G., and McLaughlin, K. A., *Nature*, **215**, 391 (1967).
[15] Fritz, O. G., and Swift, T. J., *Biophysical J.*, **5**, 675 (1967).
[16] Hazlewood, C. F., and Nichols, B. L., *Johns Hopkins Med. J.*, **123**, 198 (1968).
[17] Kavanaugh, J. L., *Water and Solute-Water Interactions*, 101 (Holden-Day, Inc., San Francisco, 1964). Emsley, J. W., Feeney, J., and Sutcliffe, L. H., *High Resolution Nuclear Magnetic Resonance Spectroscopy*, 1 (Pergamon, New York, 1965).
[18] Zierler, K. L., *Amer. J. Physiol.*, **197**, 515 (1959).
[19] Hechter, O., Wittstruck, T., McNiven, N., and Lester, G., *Proc. US Nat. Acad. Sci.*, **46**, 783 (1960).

Recognition of Bacterial Initiator *t*RNA by Initiation Factors

by
P. S. RUDLAND
W. A. WHYBROW
K. A. MARCKER
B. F. C. CLARK
MRC Laboratory of Molecular Biology, Cambridge

Factors required for initiating protein synthesis in bacteria recognize both the ribonucleic acid moiety and the presence of the formyl group in fMet-$tRNA_f^{Met}$.

THE binding of bacterial initiator *t*RNA, fMet-$tRNA_f$, to 70*S* ribosomes with the trinucleoside diphosphate ApUpG at a magnesium ion concentration of 0·005 M requires initiation factors and GTP[1-5]. Neither the unformylated species Met-$tRNA_f$ nor any other aminoacyl-*t*RNA will bind to ribosomes in such conditions. Initiation factors and GTP are also necessary for the binding of initiator *t*RNA to 30*S* ribosomal subunits[5-8]. We find that either ApUpG or GpUpG can function as the initiator triplet on these sub-particles, and that the stimulating effect of initiation factors and GTP is specific for fMet-$tRNA_f$: they fail to stimulate the binding of several other formylated aminoacyl-*t*RNAs, including fMet-$tRNA_m$ to 70*S* or 30*S* ribosomes in the presence of their respective codons. We conclude that the initiation factors specifically recognize fMet-$tRNA_f$.

Abbreviations used: the purified formylatable and nonformylatable methionine-accepting species of *t*RNA are represented by $tRNA_f^{Met}$ and $tRNA_m^{Met}$ respectively. The unformylated charged $tRNA_f^{Met}$ is Met-$tRNA_f$ and in the formylated charged state is fMet-$tRNA_f$ whereas the charged $tRNA_m^{Met}$ is Met-$tRNA_m$.

Pure methionine *t*RNAs were obtained according to Doctor *et al.*[9]. Pure [^{32}P]-labelled $tRNA_f^{Met}$ was prepared as described by Dube *et al.*[10]. The remaining *t*RNAs used in this study were obtained in an enriched form from the countercurrent distribution step in the purification of the methionine *t*RNAs. $tRNA^{Val}$ and $tRNA^{Tyr}$ were further purified by chromatography on benzoylated-DEAE cellulose and DEAE 'Sephadex' respectively. The amino-acid charging capacity of the preparations used was as follows: $tRNA^{Lys}$, 8 per cent; $tRNA^{Tyr}$, 32 per cent; $tRNA^{Val}$, 88 per cent; and $tRNA^{Phe}$, 12 per cent. Charging of *t*RNAs was as previously described[11]. *f*Met-$tRNA_f$ was prepared by charging $tRNA_f$ in the presence