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9. Ten grams of 2,2'-dinitrodiphenyl (Aldrich Chemical Co., Milwaukee, Wisconsin) were dissolved in 100 ml of red fuming nitric acid (density 1.6, Baker). The mixture was heated to 80°C, 100 ml of concentrated sulfuric acid and an additional 100 ml of red fuming nitric acid were added, and the mixture was refluxed for 1 hour. After it had cooled to room temperature, the reaction mixture was poured onto ice and water, and the product was collected by suction filtration. After six recrystallizations from boiling acetic acid, 3.37 g (29 percent yield) of pale yellow prisms were obtained, melting point 166.5° to 167.0°C (capillary, Thomas Hoover). The reported values of the melting point are 165°C [H. C. Gull and E. E. Turner, *J. Chem. Soc.* **1929**, 491 (1929)] and 165° to 167°C [(*8*); F. Ullmann and J. Bielecki, *Ber. Deut. Chem. Ges.* **34**, 2174 (1901)].
10. Absorbances were determined at 575 nm, rather than 620 nm, the maximum absorbing wavelength for the reaction, to facilitate comparisons with plant extracts at a later time. Chlorophyll interferes less with the absorbance at 575 nm than with that at 620 nm. The F peak (536 nm) of a holmium oxide filter was used to monitor the variation of the spectrophotometer at intervals throughout the experiment ($n = 39$; average absorbance, $\bar{x} = 0.313$; S.D. = 0.008).
11. The pure, crystalline calotropin-calactin mixture consisted of two isomeric cardiac glycosides (molecular weight 532.61) and was obtained from T. Reichstein. We assumed that all the cardiac glycosides in the butterflies have nearly the same extinction coefficients on reaction with TNDP. These two glycosides have been isolated from monarch butterflies reared on *Asclepias curassavica* and also occur in the leaves of this plant [T. Reichstein, *Naturwiss. Rundsch.* **20**, 499 (1967)].
12. Program 1316 EB/ST3 Rev. 1, 700 Series program library, vol. 9. Wang Laboratories, Inc., Tewksbury, Massachusetts, 1971.
13. The Ontario sample was collected between 31 August and 1 September 1970, the Massachusetts sample between 19 and 26 September 1970, the Maryland sample between 4 and 26 September 1970, and the Florida sample between 27 November and 1 December 1970.
14. According to R. E. Woodson [*Ann. Mo. Bot. Gard.* **41**, 1 (1954)], there are 12 species of *Asclepias* in northeastern North America and 21 in the Southeast. Monarchs reared on two of the latter, *A. curassavica* and *A. humistrata* Walt., are known to be extremely emetic (*1–3*).
15. Thirteen blue jays had the following dosages and responses: 0.174 g, two birds vomited; 0.157 g, four birds vomited, two did not; 0.142 g, one bird vomited, three did not; 0.129 g, one bird did not. Three additional birds at the 0.129-g dosage also did not vomit, and three birds at the 0.192-g dosage did. The 95 percent confidence levels of the ED_{50} are 0.142 to 0.163 g.
16. The ED_{50} test for the less concentrated sample broke down, probably because of the large amount of powder needed to provide a sufficient dose of toxin. More than 0.300 g of powder had to be force-fed, and apparently such large amounts of dry material interfere with absorption of the toxin. Of 14 birds tested, two vomited within 1 hour, and three within 2 hours. With lesser amounts of more toxic materials, vomiting has almost always occurred in the first hour.
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19. Supported by NSF grant GB-24888, with L. P. Brower as principal investigator. We thank Professor T. Reichstein for supplying the calotropin-calactin crystals, Dr. A. P. Platt for collecting the monarch butterfly samples in Maryland, and S. R. Kessell and J. Jaenicke for help in Florida. Drs. W. Godchaux and W. Zimmerman provided critical comments on the manuscript. We are grateful to H. Sullivan and S. C. Glazier for much help in all stages of this work and to H. Bessonette for catching and maintaining the blue jays. This report is dedicated to Dr. Virginia C. Dewey.

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An Isomorphous Heavy-Atom Derivative of Crystalline Formylmethionine Transfer RNA

Abstract. *An isomorphous osmium derivative of crystalline yeast initiator transfer RNA has been prepared and interpreted to 6-angstrom resolution. The coordinates of the heavy atoms have been determined by Patterson and "direct" methods applied to the difference coefficients of the centric projections, followed by least-squares refinement. There is one dominant site per asymmetric unit, consistent with the finding by neutron-activation analysis that there is approximately one osmium atom per molecule of transfer RNA. The osmium derivative appears to be a normal substrate for enzymatic aminoacylation.*

A complete understanding of transfer RNA (tRNA) structure will almost certainly require a crystallographic solution based on the method of heavy-atom isomorphous replacement. We report the preparation of a useful "single-site" osmium derivative of yeast formylmethionine tRNA (yeast Os-tRNA^{fMet}) which has been interpreted in crystallographic terms. Initial chemical studies indicate that one osmium atom is bound irreversibly to the tRNA molecule at a site that does not interfere with enzymatic aminoacylation.

Crystals of yeast initiator tRNA (yeast tRNA^{fMet}) were reacted with a 100-fold excess of osmium by soaking in $\simeq 0.3$ ml of stabilizing supernatant solution (*p*H 5.50) composed of 2.90*M* $(NH_4)_2SO_4$, 0.005*M* cacodylate and Mg^{2+}, 0.002*M* spermine, 0.002*M* K_2OsO_4 (Alfa Inorganics), and 0.062*M* pyridine. The crystals gradually became colored, and appeared blue when viewed down the hexagonal axis and amber when viewed normal to the unique axis. The color change appeared to be complete within 2 weeks. No loss of color was noted when the Os-tRNA^{fMet} crystals were soaked for several months in an osmium-free supernatant solution.

Table 1. Neutron-activation analysis of crystalline yeast Os-tRNA^{fMet}. Sample (a) was a crystal soaked for at least 7 days in osmium-free supernatant solution, then dissolved in water. Sample (b) received the same treatment as sample (a) but was also passed over G-25 Sephadex that was equilibrated with a solution of 0.05*M* $(NH_4)_2SO_4$, 0.005*M* Mg^{2+}, and 0.005*M* cacodylate, *p*H 5.6. Sample (c) received the same treatment as sample (a) but was also dialyzed against a solution of 0.2*M* NaCl, 0.001*M* ethylenediaminetetraacetic acid, 0.005*M* formic acid, and 1 percent ethylene glycol at *p*H 4.0; then passed over G-25 Sephadex that had been first equilibrated with the same solution. Sample (d), the control, was a dissolved crystal that was not exposed to osmium. The A_{260} unit has been defined (*5*) and is taken to equal 2.0 nmole of yeast tRNA^{fMet}. The precision of osmium analysis at this level is 3.3 percent. (The analysis was performed by G. Reed.)

Sample	A_{260} units	Osmium	
		10^{-7} g	Gram-atoms per mole of yeast tRNA
a	0.94	3.84	1.1
b	0.35	1.35	1.0
c	0.60	2.24	1.0
d	2.50	0.00	0.0

The stoichiometry of the complex was established by neutron-activation analysis. Table 1 shows that one osmium atom was bound per molecule of tRNA—even after the tRNA was "denatured" and subjected to gel filtration over G-25 Sephadex that had first been equilibrated with a denaturing buffer containing 1 percent (by volume) ethylene glycol to scavenge Os VI (*1*). Thus the binding is apparently "irreversible," a result suggesting that the osmium is either covalently bound or very tightly enmeshed in the secondary structure.

Since Os VI • 2pyridine is known to form stable adducts with *cis*-diol groups (*1*) (indeed, this was the rationale behind trying the reaction), the yeast Os-tRNA^{fMet} crystals were dissolved and tested to see whether or not the terminal 3'OH was free to accept methionine. Yeast Os-tRNA^{fMet} accepted 1.45 nmole of methionine per unit of absorbance at 260 nm (A_{260} unit), whereas tRNA^{fMet} obtained from parent crystals accepted 1.55 nmole of methionine per A_{260} unit. Since the difference in acceptance between the derivative and parent is less than the differences normally encountered between various batches of crystalline tRNA^{fMet}, it is clear that under typical assay conditions (*2*) the Os-tRNA^{fMet} could be enzymatically charged to the same extent as the parent molecule. In view of the firmness with which osmium is bound, we conclude that the 3'OH terminus is not the primary site of osmium attachment. Although a Michaelis constant has not yet been determined for the yeast Os-tRNA^{fMet} adduct, rapid and full enzymatic acylation of Os-tRNA^{fMet} suggests that the site of osmium attach-

ment is not important for synthetase binding.

The reaction of osmium with nucleic acids and their monomeric components has been studied (*3*). Stable adducts, particularly with thymine, can be formed in the presence of pyridine. It is tempting to speculate that the single thymine residue present in most tRNA molecules represents the dominant binding site, but this must await the sequence determination of yeast $tRNA^{fMet}$ and identification by chemical means of the osmium binding site in the primary structure.

The crystals are hexagonal in the space group $P6_222$ (or $P6_422$), with unit cell dimensions $a = b = 115.3$ Å and $c = 136.9$ Å. There are 12 molecules in the cell, one per asymmetric unit. Solvent comprises 82.5 percent of the cell volume (*4, 5*). Crystals of yeast Os-$tRNA^{fMet}$ are as well ordered as the native crystals and appear to be isomorphous, with no change in the *a* axis and a 0.3 percent increase in the *c* axis.

The crystallographic analysis was carried out to 6-Å resolution by conventional precession photography with CuKα radiation reflected from a graphite monochromator. Intensities were measured by a flying-spot densitometer (*6*). The average difference between intensities of the osmium derivative and parent was 20 percent, compared with a mean difference of 8 percent between intensities from identical planes of two different native crystals.

A difference Patterson map of the (*0kl*) projection is shown in Fig. 1a. The interpretation of this map as well as that of the ($2h\ \bar{h}l$) and (*hk0*) projections is consistent with a single dominant site per asymmetric unit having the refined coordinates given in the following discussion.

In space groups of high symmetry the interpretation of a Patterson synthesis can present a formidable problem—particularly in projection. In the space group $P6_222$, the simplest case of one heavy atom per asymmetric unit involves deconvoluting 33 interatomic vectors represented by 21 peaks. Multiple sites compound the difficulty seriously. Steitz (*7*) introduced the use of "direct methods" to locate heavy atoms in the centric projections of protein crystals. The E_Δ map (*8*) produced by such an analysis has many fewer peaks and requires no deconvolution, because it is not a vector map but rather a direct (and sharpened) representation of the heavy atoms. Figure 1b shows the E_Δ map of the heavy atoms of this derivative viewed in the (*0kl*) projection. This map was derived by application of the Σ_2 symbolic addition procedure to the normalized moduli of the difference amplitudes. The three peaks observed in the asymmetric unit of this projection are not constrained by the form of the calculation or by the symmetry of the coefficients to bear any symmetrical relationship to one another. The fact that the peaks occur at positions related by the three-dimensional space group symmetry lends considerable credibility to the map.

The interpretation of the difference maps was checked by considering peaks A, B, and C of Fig. 1c as independent sites in the plane group *pmm* and calculating phases for the parent structure factors on the basis of sites A and B only. In effect, this treats site C as if it were an unknown subsidiary site. If sites A and B are correctly interpreted, then the phases of the parent structure

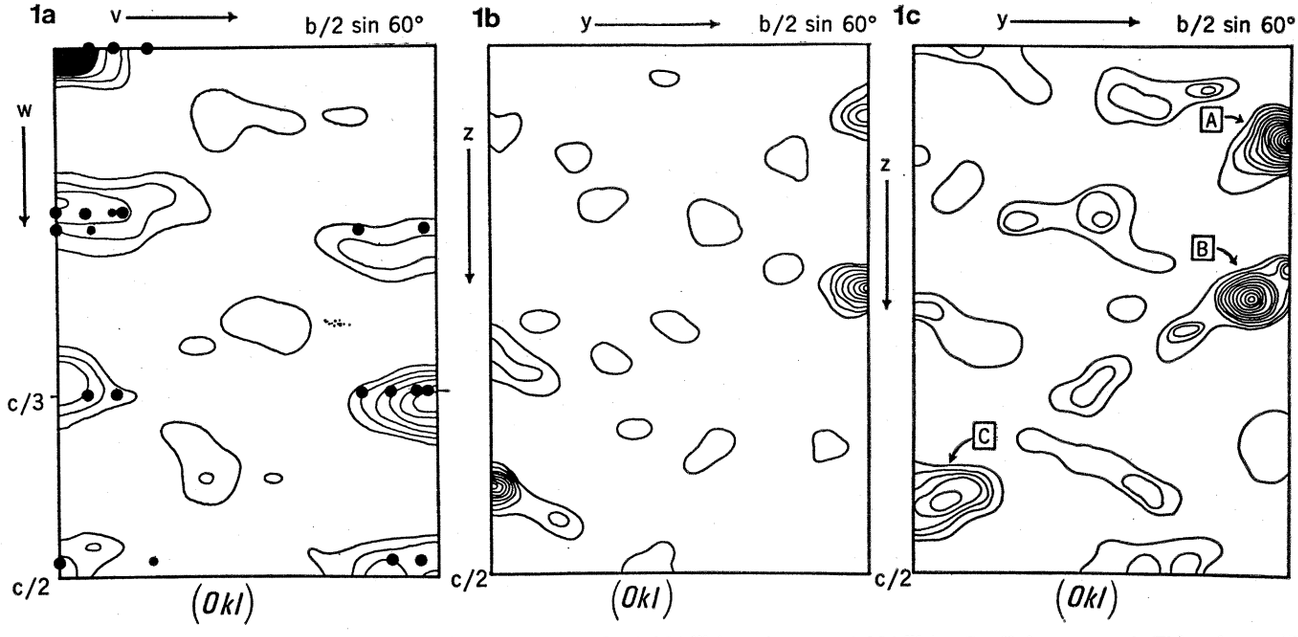

Fig. 1. (a) Difference Patterson projection between yeast Os-$tRNA^{fMet}$ and yeast $tRNA^{fMet}$. Coefficients are $(\Delta F)^2$, where ΔF is the difference in structure amplitude between the osmium derivative and the parent (P), that is, $(|F_{Os+P}| - |F_P|)$. Peaks expected for a single osmium atom having the coordinates (.036, .476, .091) are indicated by ●, double-weight vector; •, single-weight vector. (b) E_Δ map of the osmium atoms in projection. The coefficients are E_Δ, where $|E_\Delta|^2 = |\Delta F|^2/\epsilon^2 \sum_j^N f_j^2$; f_j is the scattering factor of the *j*th osmium atom of the unit cell and ϵ is a symmetry factor (*13*). The sign of $E_\Delta(h)$ is given by the Σ_2 relation (*14*), sign of $[E_\Delta(h)] \approx$ sign of $[\sum_{h'} E_\Delta(h')\, E_\Delta(h - h')]$, which was evaluated by the symbolic addition procedure (*15*) as programmed by Dewar (*16*). For the space group $P6_222$, peaks are expected in the asymmetric unit of the (*0kl*) projection at A = *y*, *z*; B = $x - y$, $\frac{1}{3} - z$; C = *x*, $\frac{1}{3} + z$. These positions correspond closely to the three dominant peaks of the figure when values for *x*, *y*, and *z* from Fig. 1a are used. (c) (*0kl*) difference Fourier projection between yeast Os-$tRNA^{fMet}$ and yeast $tRNA^{fMet}$. The coefficients are $\Delta F \cos \phi_{AB}$, where ΔF is defined in Fig. 1a and ϕ_{AB} is the phase (0 or π) for the parent, based on sites A and B *only*. Only terms for which the figure of merit is 0.5 or greater were used. For this calculation, A, B, and C were considered independent sites in the plane group *pmm*.

should be sufficiently accurate to find site C by a difference Fourier map. The strong peak at site C in Fig. 1c appears with the expected reduction in peak height (*9*).

The heavy-atom parameters were refined to 6-Å resolution in the (*0kl*) projection with all coordinates constrained by $P6_222$ symmetry. The least-squares procedure and isomorphous phase calculation were essentially as described by Dickerson *et al.* (*10*); a program by Adams *et al.* (*11*) was used. A single site of 49 electrons gave refined coordinates of $x = .036$, $y = .476$, and $z = .091$, with a combined form-temperature factor of 278. Since more than 80 percent of the cell volume is solvent, it is reassuring that the osmium binding site is within the region indicated by various molecular search studies to be occupied by tRNA molecules (*4, 12*). The R factor:

$$\frac{\sum_h \left| \, |\Delta F(h)| - |F_{Os}^{calc}(h)| \, \right|}{\sum_h |\Delta F(h)|}$$

was 0.69, which is unexpectedly high in view of the close correlation between the E_Δ map of the (*0kl*) projection and the self-consistent interpretations of the various difference Patterson projections. The large residual may be due to subsidiary sites, because (i) certain peaks consistently recur in both the difference Fourier and Patterson projections, and (ii) there is only two-thirds occupancy of the primary site despite an overall stoichiometry of one osmium atom per tRNA molecule. Efforts to unambiguously establish the positions of subsidiary sites have not been successful in projection, and their determination awaits the analysis of the three-dimensional data.

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Brain and Body Temperatures in a Panting Lizard

Abstract: *Panting in* Sauromalus obesus *is effective enough to keep deep body temperature (T_C) and brain temperature (T_B) below an ambient temperature of 45°C for extended periods of time and has a greater cooling effect on the brain than on the remainder of the body. Six animals maintained T_C and T_B 0.9°C (± 0.08 standard error) and 2.7°C (± 0.2 standard error) respectively lower than the ambient temperature of 45°C. It is possible that intracranial vascular shunts play a role in cranial cooling during panting.*

A number of investigators have pointed out the similarity between panting in homeothermic animals and the ventilatory response of certain desert lizards to heat stress, with the implication that the latter is a thermoregulatory response (*1*). Crawford and Kampe (*2*) suggested that the increase in respiratory ventilation could be due to the increase in oxygen consumption which occurs when the body temperature of a lizard is raised. They attempted to separate the respiratory response of the lizard *Sauromalus obesus* into its metabolic and possible thermoregulatory components. Although the respiratory pattern and evaporative cooling of *Sauromalus* at a body temperature of 43.5°C was consistent with the panting hypothesis, the animals did not dissipate heat sufficiently to maintain body temperature below that of the environment (43°C). Crawford and Kampe (*2*) therefore concluded that panting was of marginal importance in the overall heat balance of the animal.

It was necessary, in their experiments, to confine the animal in a double chamber which separated the head from the remainder of the body so that respiratory and cutaneous evaporation could be determined separately and simultaneously. Since it is unclear whether a seal separating the head from the remainder of the body interferes with respiration or gular flutter, it is desirable to determine whether *Sauromalus*, unencumbered by experimental apparatus, can maintain a body temperature less than that of the environment. Furthermore, the carotid arteries of *Sauromalus* run so close to the surface that they are visible in the pharynx, and they enter the cranium from an area exposed to air movement, particularly during gular flutter. It is possible that during panting and gular flutter evaporation from this area cools the carotid blood as it enters the cranial cavity, serving primarily to cool the brain rather than the entire body. Experiments were therefore designed to measure brain and deep body temperature simultaneously.

Animals (*S. obesus*) were anesthetized with tricaine methanesulfonate (MS-222; Ayerst), 400 mg/kg. A small hole, 1 mm in diameter, was drilled through the skull 1 to 2 mm behind the pineal organ. Membranes surrounding the brain were not penetrated by this procedure. The animals were allowed to recover overnight and appeared to be "normal" the following day. Brain thermocouples were prepared from 40-gauge copper and constantan wire. Thermocouples were passed through a 0.5-cm² Lucite plate, insulated and stiffened with a thin layer of dental acrylic, and implanted in unanesthetized lizards to a depth of about 0.5 cm. The position of the thermocouples, determined at autopsy, was at the rostral