

Multiple steps in DNA recognition by restriction endonuclease from *E. coli* K

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The process of DNA recognition by the activated form of the restriction endonuclease from *E. coli* K involves three enzyme-DNA complexes which can be differentiated experimentally. These are: an initial complex formed at a nonspecific site; a recognition complex involving the host specificity site; and a cleavage complex dependent on the presence of ATP.

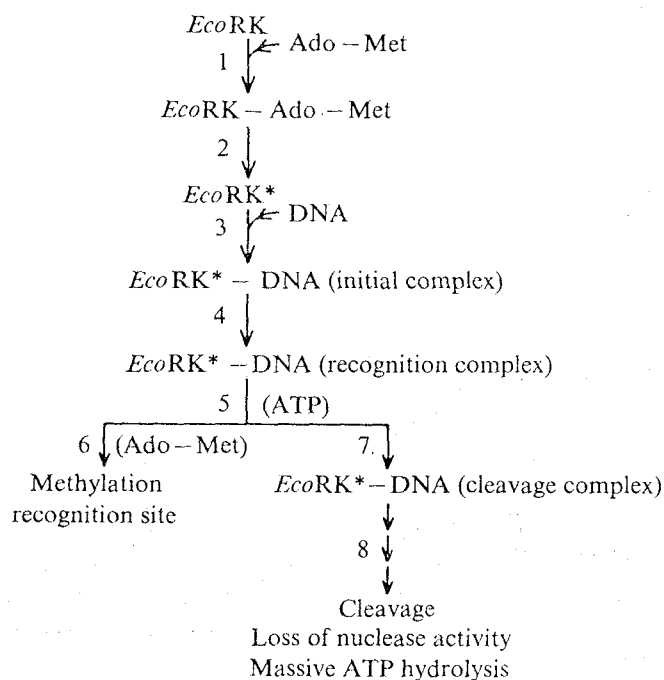
THE restriction endonuclease from *Escherichia coli* K, *Eco*RK, is a complex multifunctional protein which cleaves unmodified DNA in the presence of S-adenosylmethionine (Ado-Met), ATP and Mg²⁺ (ref. 1). A powerful ATPase activity appears during the course of this restriction reaction² and the same protein is also able to methylate unmodified DNA, rendering it insensitive to its own restriction activity³.

The way in which the enzyme recognises the host specificity sequence on the DNA, and the factors that determine whether it will act as an endonuclease, or as a methylase, are two aspects of the mechanism which go beyond the immediate area of restriction and modification. The first of these, recognition of nucleotide sequences, is common to most proteins that react with nucleic acids. The second is that the interaction involved in the recognition of the nucleotide sequence directs the protein towards either of two opposed modes of action, restriction or modification. In other words, a specific nucleotide sequence may not only be recognised by a protein, but also act as a signal for the mode of action of a multifunctional enzyme.

In a certain perverse way, it is the very complexity of this protein and the reactions that it carries out that enables us to distinguish experimentally four major stages in the *in vitro* reaction (Fig. 1). These are: Enzyme activation: *Eco*RK binds Ado-Met rapidly, and in a slow second step, an allosteric transition to an activated form (*Eco*RK*) takes place. DNA recognition: *Eco*RK* interacts with DNA, first, at a nonspecific site (initial complex), and then at the unmodified recognition site (recognition complex). The recognition site is the host specificity site on the DNA that confers susceptibility to a given restriction and modification system (the sites for the K system are called sK). Enzyme action: in the absence of methyl groups at the recognition site, the DNA will be either cleaved or methylated. Methylation occurs at the recognition site while DNA scission takes place elsewhere. Loss of nuclease activity: the enzyme is altered following DNA cleavage, and is unable to cleave again. This phenomenon is probably related to the appearance of a vigorous and sustained ATP hydrolysis which begins at the time of DNA cleavage, but continues long after the DNA digest has reached its limit.

This paper deals with the process of DNA recognition. *Eco*RK* can form three distinct complexes with DNA in its restriction mode. It first forms an initial complex at a nonspecific site which is transformed to a more stable recognition complex if an unmodified host specificity site is present on the DNA. Neither of these complexes can be trapped on membrane filters. If ATP is added to the recognition complex, a rapid transition to a third complex which can be trapped on filters, takes place. This presumably occurs at the cleavage site.

Fig. 1 Reaction mechanism of endonuclease *Eco*RK.



Enzyme forms a specific complex with unmodified DNA that is retained on filters

*Eco*RK has previously been shown to form a specific complex with unmodified λ .0 DNA that can be stabilised with EDTA and detected by binding to nitrocellulose filters. (λ bacteriophage grown on a non-modifying host is designated λ .0; phage grown on a host that confers K-specific modification to the DNA is called λ .K.) The formation of this complex requires the presence of Ado-Met, ATP and Mg²⁺ (ref. 4). No filter-binding complex can be detected with modified λ .K DNA or with DNA that contains no recognition sites for the enzyme. Complex formation reaches its maximum after 2 min at 30 °C, and then decays at a rate consistent with its dissociation after DNA cleavage.

That this complex was not the result of binding to sK sites on the DNA was suggested by experiments with a purified restriction endonuclease from a mutant strain of *E. coli* K12 (ref. 5). This strain is unable to restrict, but can modify normally. As expected, the restriction enzyme purified from it can methylate (but cannot cleave) unmodified DNA *in vitro*. It does not form the filter binding complex. Therefore, *Eco*RK can recognise and methylate λ .0 DNA without forming a filter binding complex. The conclusion then is that the filter-binding complex must be associated with the cleavage reaction, and not DNA recognition proper.

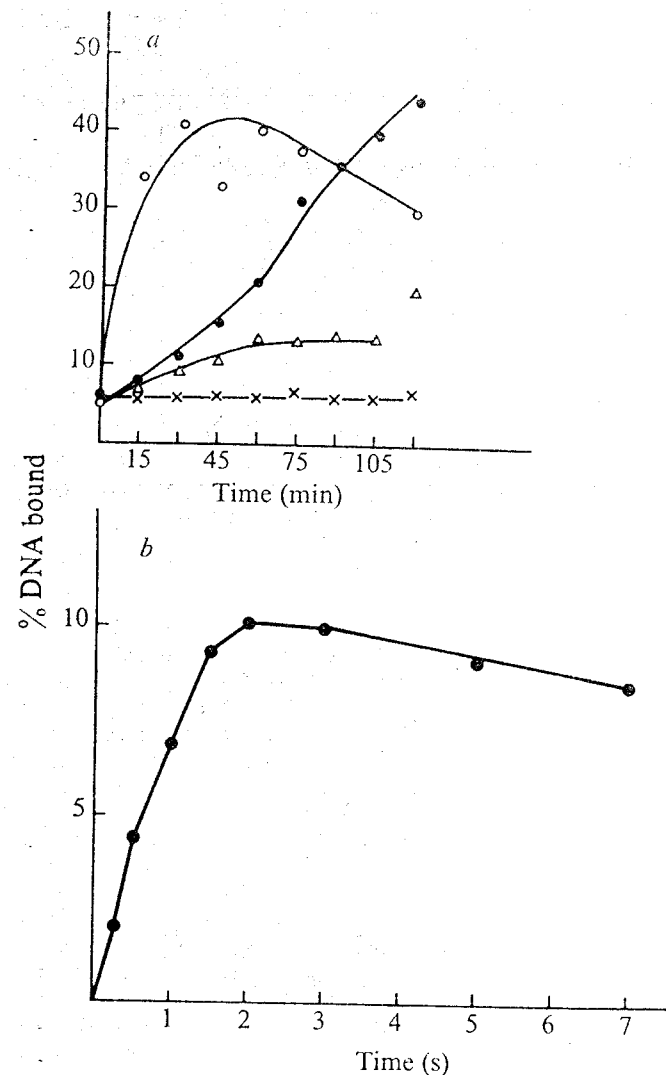


Fig. 3 Kinetics of enzyme-DNA interactions. *a*, Kinetics of DNA binding to filters after ATP addition. *b*, Kinetics of enzyme activation in presence of DNA. *a*, Standard reaction kinetics were followed by adding 9 μ l *EcoRK* (1.8 μ g) to a prewarmed reaction mixture containing in 4.5 ml: 1.6 μ g λ .0 32 P-DNA (2.16×10^5 c.p.m.), 1.8 μ mol ATP and 120 nmol Ado-Met. Aliquots (250 μ l) were removed into 25 μ l 0.5 M EDTA after different times of incubation at 30 $^{\circ}$ C and the samples were then assayed on filters. *EcoRK** was formed by adding 18 μ l *EcoRK* (3.6 μ g) to 190 μ l reaction mixture containing 1 nmol Ado-Met and incubating for 3 min at 30 $^{\circ}$ C. This was chilled in ice and 5 μ l aliquots were added to 250 μ l reaction mixture containing 0.09 μ g λ .0 32 P-DNA (1.2×10^4 c.p.m.) and 0.1 μ mol ATP. Incubation was carried out for varying time intervals, the reaction was stopped with EDTA and the samples were filtered. In a separate set of incubations, 5 μ l of the same *EcoRK** was added to 250 μ l of a similar mixture, but without ATP, the incubation continued for 3 min and 0.1 μ mol ATP was then added. After further incubation at 30 $^{\circ}$ C for varying time intervals, the reactions were terminated with EDTA and filtered. In the last set of reactions, the *EcoRK**- λ .0 DNA recognition complex was formed by adding 9 μ l (1.8 μ g) *EcoRK* to 90 μ l reaction mixture containing 2.4 nmol Ado-Met and 1.6 μ g λ .0 32 P-DNA (2.16×10^5 c.p.m.) and incubating for 3 min at 30 $^{\circ}$ C. The mixture was chilled in ice and 5 μ l aliquots were added to 250 μ l reaction mixture containing 0.1 μ mol ATP. The samples were incubated for different time intervals at 30 $^{\circ}$ C, stopped with EDTA and filtered. *b*, Reaction mixture (60 μ l) containing 2.75 μ g λ .0 32 P-DNA (2.2×10^5 c.p.m.), 0.13 nmol Ado-Met and 1 μ l (0.2 μ g) *EcoRK* was incubated at 30 $^{\circ}$ C, and at different times 3 μ l aliquots were removed and mixed with 200 μ l prewarmed buffer containing 0.1 μ mol ATP. Each sample was then incubated for a further 2 min, 50 μ l 0.5 M EDTA was added and the samples were filtered and counted as before. ●, Standard reaction kinetics; △, kinetics with *EcoRK**; ×, incubation of *EcoRK** with DNA in absence of ATP; ○, kinetics of filter binding with preformed recognition complex.

the rate when *EcoRK** is added to a preformed complex. Dissociation of the enzyme from such initial complexes must therefore be a relatively rapid process and the activated enzyme must interact with many different DNA molecules during protection by non-substrate DNA (compare the half life for protection of 6.5 min with the rapid release detected above).

Recognition complex is insensitive to heparin

The polyanionic glycan, heparin, is an inhibitor of both the DNA binding and cleavage reactions (R. Y., T. A. B., W. E., and C. B., unpublished). This inhibition has been used to study the interactions between the enzyme and substrate DNA. Table 1 shows the results of one such series of experiments. The addition of

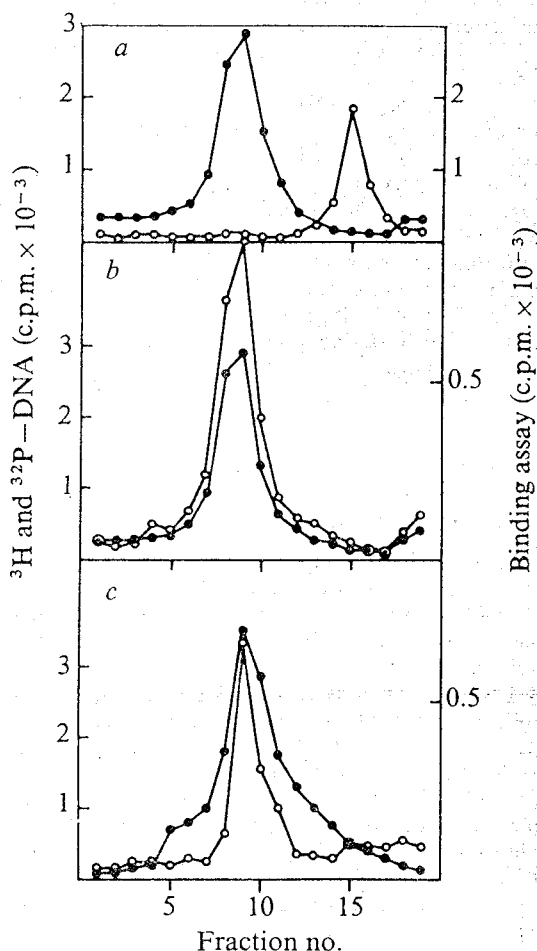


Fig. 4 *EcoRK**-DNA complexes on glycerol gradients. *a*, Composite showing the positions of *EcoRK** and λ DNA. *b*, *EcoRK**- λ .0 DNA complex. *c*, *EcoRK**- λ .K DNA complex. Four reaction mixtures of 100 μ l containing: *a*, 0.45 μ g λ .0 32 P-DNA (63,000 c.p.m.); *a*, 6.7 nmol Ado-Met and 5 μ l (1 μ g) *EcoRK*; *b*, 0.45 μ g λ .0 32 P-DNA (63,000 c.p.m.), 6.7 nmol Ado-Met and 5 μ l (1 μ g) *EcoRK*; *c*, 0.57 μ g λ .K 3 H-DNA (132,000 c.p.m.), 6.7 nmol Ado-Met and 5 μ l (1 μ g) *EcoRK*. All reactions were incubated at 30 $^{\circ}$ C for 3 min, chilled in ice and layered on 4 ml 10-30% glycerol gradients containing 20 mM potassium phosphate, pH 7.0, 5 mM 2-mercaptoethanol and 0.2 mM EDTA. These were centrifuged at 55,000 r.p.m. for 100 min at 4 $^{\circ}$ C in an international SB 405 rotor. Twenty fractions were collected from each gradient and 50 μ l aliquots were removed from each fraction from *a*, *b* and *c* for counting. Aliquots (50 μ l) of *a* and *c* were assayed by the standard procedure except that Ado-Met was omitted from the incubation. Aliquots from *b* were assayed similarly except that both Ado-Met and additional λ .0 32 P-DNA were omitted.

Table 1 Inhibition by heparin of DNA binding to filters by *Eco*RK

First incubation	Second incubation	λ .0 32 P-DNA bound (c.p.m.)	% Maximum
Set 1			
1 Complete	—	6,565	100
2 Complete+heparin	—	5,569	84.8
		80	1.2
		70	1.1
3 Complete—enzyme	—	46	0.7
		55	0.8
Set 2			
4 <i>Eco</i> RK + Ado-Met	λ .0 DNA + ATP	2,185	86
		2,541	100
5 <i>Eco</i> RK + Ado-Met	λ .0 DNA + ATP	46	1.8
+ heparin		35	1.4
6 <i>Eco</i> RK + Ado-Met	λ .0 DNA + ATP + heparin	35	1.4
		77	3.0
Set 3			
7 <i>Eco</i> RK + Ado-Met + λ .0 DNA	ATP	1,682	87.9
		1,913	100
8 <i>Eco</i> RK + Ado-Met + λ .0 DNA	ATP	36	1.9
+ heparin		24	1.3
9 <i>Eco</i> RK + Ado-Met + λ .0 DNA	ATP + heparin	800	41.8
		1,131	59.1

Set 1, Each reaction mixture of 250 μ l contained 0.13 μ g λ .0 32 P-DNA (14,650 c.p.m.), 6.7 nmol Ado-Met and 0.1 μ mol ATP. Sample 2 also contained 0.25 μ g heparin. *Eco*RK (0.1 μ g, 0.5 μ l) was added to the samples indicated and incubation at 30 °C was carried out for 2 min. The reaction was stopped by the addition of 50 μ l 0.5 M EDTA and filtered.

Set 2, Each reaction mixture of 5 μ l contains 27 pmol Ado-Met. In sample 5, 0.25 μ g heparin was also present. *Eco*RK (0.5 μ l, 0.15 μ g) was added, the samples were incubated at 30 °C for 3 min and diluted with 250 μ l of a reaction mixture containing 0.1 μ mol ATP and 0.13 μ g λ .0 32 P-DNA (14,650 c.p.m.) per 250 μ l or a similar mixture containing in addition 0.25 μ g heparin. After a further 2 min incubation the reaction was stopped with EDTA and the samples filtered.

Set 3, Procedure as for set 2 except that the λ .0 32 P-DNA was incubated with the 5 μ l enzyme activation mixture rather than added later during the dilution step.

heparin to a standard reaction mixture abolishes the binding of DNA to filters (Table 1, set 1). Heparin also inhibits the activated enzyme, both when it is added to the activation mixture, and when it is added to a dilution buffer containing DNA and ATP (Table 1, set 2). If, however, the enzyme is activated in the presence of λ .0 DNA and then diluted into buffer containing ATP and heparin, little inhibition is observed (Table 1, set 3). If heparin is added to a recognition complex, and ATP is added later, the same lack of inhibition is observed. Therefore, once the recognition complex has formed, the enzyme is no longer sensitive to inhibition by heparin. Normal DNA cleavage is observed when ATP and heparin are added to such a complex.

Both the initial and recognition complexes can be isolated on glycerol gradients

Direct evidence for the various enzyme-DNA complexes was obtained by glycerol gradient centrifugation. Sedimentation through glycerol gradients readily resolves DNA from the free enzyme (Fig. 4a, a composite of two different gradients). To detect the recognition complex, *Eco*RK was incubated with λ .0 32 P-DNA, Ado-Met and Mg^{2+} at 30 °C, chilled and sedimented through a glycerol gradient. Aliquots from each fraction were counted to determine the position of the DNA and further aliquots were assayed for the recognition complex (Fig. 4b). To detect the initial complex, the experiment was repeated with λ .K 3 H-DNA in place of λ .0 DNA, and the gradient was assayed after addition of ATP and λ .0 32 P-DNA to an aliquot from each fraction (Fig. 4c). In both cases, the activated enzyme comigrated with the DNA. Surprisingly, a control incubation of enzyme with λ .0 32 P-DNA showed that the endonuclease was also bound to the DNA in spite of the fact that it had not been activated with Ado-Met (data not shown). This particular complex, however, does not seem to be an intermediate in the restriction reaction as the binding kinetics of this complex after ATP and Ado-Met addition are those of a standard reaction. In fact, by addition of *Eco*RK to a λ .0 32 P-DNA complex in a labelled state, the reaction is inhibited by heparin.

An enzyme mechanism

Steps 1 and 2 of the reaction mechanism (Fig. 1) have been described in detail elsewhere⁸, and involve the fast binding of Ado-Met by the enzyme followed by a slow transition to an activated form.

Here, we are primarily concerned with the phenomenon of DNA recognition which we show involves the sequential formation of at least three different types of enzyme-DNA complex. To understand this process, we must briefly describe the relationship between the sites on the DNA for host specificity, methylation and cleavage. The recognition sites are those sites on the DNA that confer susceptibility to the endonucleolytic action of the enzyme. They are relatively few in number for a given DNA and may be lost by mutation to yield a DNA molecule that cannot be cleaved, *in vitro* or *in vivo*, by the enzyme⁹. The methylation site seems to be a specific sequence of bases at the recognition site, since phage mutants that have lost their recognition sites also lose (again, both *in vivo* and *in vitro*) the ability to be methylated^{9,10}. Furthermore, Horiuchi *et al.* have isolated small, specific DNA fragments from modified f1 DNA and found that of these fragments, only the two that contain the genetically mapped sB sites were methylated¹¹. The situation concerning the cleavage sites is less clear. The enzyme does not cleave the DNA at the recognition sites and the number of possible cleavage sites is larger than the number of recognition sites¹². Mapping studies with PM2 DNA have shown that the K cleavage sites are related to the readily denaturable regions of the genome (R.Y., T.A.B., W.E., and C.B., unpublished).

The interaction of *Eco*RK* with the host specificity site presents one of the most interesting aspects of the reaction mechanism because it is this interaction which will determine whether the enzyme will methylate or cleave the DNA. The site can exist in three possible forms: fully modified, fully unmodified or heteroduplex (one strand modified, the other not). We have previously shown that such heteroduplex DNA is not a substrate for modification, and *Levy et al.*¹³ have shown that it is the best substrate for methylation and that Ado-Met, ATP and

the fact depends on whether the restriction enzyme recognises the site if the site is unmodified, it will trigger the enzyme to its restricting mode, the enzyme will proceed to a cleavage site and restrict the DNA. If it is modified on one strand, the enzyme will be altered to its modifying form, and methylate the other strand. If it is fully modified, the enzyme will not recognise it, and will continue to scan other DNA molecules. The interactions of the enzyme with heteroduplex DNA are currently the object of extensive study in our laboratory. Both the recognition and cleavage complexes protect a small segment of DNA from DNase digestion. Sequencing of these fragments is currently in progress, and should clarify the location on the DNA of the recognition and cleavage complexes.

The restriction enzyme first breaks one strand followed some time later by a break on the opposite strand¹. At this point, it no longer behaves as an enzyme, since having cleaved once, it does not do so again. Simultaneously with DNA cleavage, a vigorous ATP hydrolysis occurs, which continues for a long period of time². Both observations can be readily reconciled if the restriction enzyme dissociated during cleavage to yield a species that would remain bound to the DNA. This complex would catalyse the ATP hydrolysis. Three observations lend support to this view: detection of a stable protein-DNA complex that hydrolyses ATP¹⁴; the lack of turnover (measured by both DNA binding and cleavage); and the complementation of a limit digest by either of two mutant restriction enzymes⁵ to give a new round of endonuclease activity.

It is possible that the restriction enzyme is a complex of several subunits, each with its own separate machinery to carry out reactions that are carried out in a much simpler manner by other restriction and modification systems. It cannot be ruled out that the complexity may be related to its involvement in other reactions such as DNA replication or recombination.

This work was supported in part by the Swiss National Foundation for Scientific Research. We thank Drs W. Arber and V. Pirrotta for comments on the manuscript, and Drs G. Vovis, K. Horiuchi and N. Zinder for making available their results before publication.

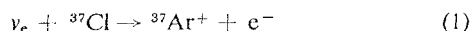
Received May 5; accepted June 23, 1975.

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letters to nature

Chemistry of the solar neutrino problem

ELECTRON neutrinos with energies of about 1-10 MeV are generally presumed to be produced by thermonuclear reactions in the Sun's core. Since 1967 an experiment has been under way on Earth to detect a subset of these neutrinos: argon atoms from the reaction



are chemically isolated, and the Auger electrons characterising their electron-capture decay are counted.¹ A sizeable discrepancy—the solar neutrino problem^{2,3}—has appeared. The most recent observational results⁴ imply an upper limit (1σ) of 1 SNU ($= 10^{-36}$ solar neutrino captures per target ${}^{37}\text{Cl}$ nucleus s^{-1}), whereas the latest theoretical expectation based on standard models⁵ is 5.6 SNU.

Something is definitely awry in our understanding of solar structure theory, nuclear physics, neutrino physics, or the detection chemistry. A solution to the solar neutrino problem has been extensively sought in the first three of these areas^{6,7} (see also refs 2 and 3), but these efforts have recently encountered severe constraints⁸⁻¹⁰. The last area—the chemistry of the detection experiment—is one of the more neglected aspects of the problem; here I shall attempt to rectify this imbalance.

In the solar neutrino experiment a tank of liquid tetrachloroethylene (C_2Cl_4) provides the target ${}^{37}\text{Cl}$ nuclei of equation (1). The number of ${}^{37}\text{Ar}$ nuclei in the tank, $N(t)$, is governed by the equation

$$dN/dt = R + b - (N/\tau) \quad (2)$$

where R and b are the respective production rates resulting from solar neutrinos and background processes, and where τ ($= 50.6$ d) is the electron-capture-decay lifetime of ${}^{37}\text{Ar}$. For $t \gg \tau$, the solution to equation (2) asymptotically approaches the limiting value, $N(\infty) = (R + b)\tau$. The detector parameters¹ yield $R\tau = 9.6$ ${}^{37}\text{Ar}$ nuclei per SNU; the cosmic-ray-induced background for the water-shielded tank is estimated¹¹ to be $b = 0.09$ ${}^{37}\text{Ar}$ nuclei d^{-1} . Therefore

$$N(\infty) = 9.6(\text{SNU}) + 4.5 \quad (3)$$

where (SNU) denotes the actual capture rate of solar neutrinos in the tank of C_2Cl_4 .

Standard theory⁵ implies that (SNU) $\simeq 5.6$, so the anticipated steady-state population from equation (3) is $N(\infty) \simeq 58$ ${}^{37}\text{Ar}$ nuclei. The experimenters are attempting to extract fewer than 100 ${}^{37}\text{Ar}$ atoms from 390,000 l of tetrachloroethylene, to count them, and to determine the number produced by solar neutrinos in comparison with that arising from background processes. The complexity and marginal nature of the experiment lead me to put forward the hypothesis that the solar neutrino problem is solely an artefact of the chemistry of the detection technique. A direct corollary is that ${}^{37}\text{Ar}$ nuclei are, in fact, being produced by solar neutrinos in the tank at the rate of 5.6 SNU, so that $N(\infty) = 58$ ${}^{37}\text{Ar}$ nuclei. The experimental upper limit of 1 SNU then implies that only a small fraction of the ${}^{37}\text{Ar}$ in the tank is revealed in the output pulses of the Auger counter. (I shall use the loose terminology, 'chemical trapping', to indicate the fate of the undetected ${}^{37}\text{Ar}$ nuclei.)

Five experiments constrain this chemical hypothesis. First,¹² a known volume of pure ${}^{36}\text{Ar}$ gas was dissolved in the tank