A curve calculated from model B ($n = 3$) is shown in Fig. 1. This curve fits the experimental points substantially better than those from models A_2 and A_3 (all have three arbitrary constants). An almost equally good fit can be obtained with model B when $n = 4$ (with adjustment of the other constants), but values of $n = 2$ or 5 will not give a satisfactory fit. The fit of model A_3 can be slightly improved (at the expense of additional arbitrary constants) by giving the three sites different microscopic binding constants, but the fit of model B remains substantially better. Model B is also the most attractive in the sense that the Ca^{2+}-independent light is an integral part of the model rather than simply an added constant. More detailed structural and chemical information about the aequorin molecule will be required to finally decide among these and other possible models.

The results are important to the use of aequorin as an intracellular calcium indicator. Clearly in the range of $[Ca^{2+}]$ where the Ca^{2+}-independent light becomes a significant fraction of the Ca^{2+}-dependent light, the utility of aequorin as a $[Ca^{2+}]$ indicator will be correspondingly reduced. Under the conditions of the experiment in Fig. 1, $[Ca^{2+}]$ below about $3 \times 10^{-8} M$ could not be determined with aequorin. However, this limiting concentration is crucially dependent on the conditions; for instance, we have found that a change in KCl concentration from 0.01 to 0.50*M* produces an approximately parallel shift of the curve to the right by 1.5 log units. In addition, there is a change in the level of Ca^{2+}-independent light (noted above). As an overall consequence the minimum detectable $[Ca^{2+}]$ at 0.50*M* KCl might be about $10^{-7} M$, but at 0.01*M* it might be $10^{-9} M$. The $[Mg^{2+}]$ and perhaps other aspects of the intracellular environment will also affect the minimum detectable $[Ca^{2+}]$.

The fact that the slope of the relation between light and $[Ca^{2+}]$ varies continuously with $[Ca^{2+}]$ introduces further difficulties into the use of aequorin as a quantitative $[Ca^{2+}]$ indicator. Previously we (*4*) and others (*8*) felt that one of the most practicable approaches to calibrating the aequorin response in vivo was to determine the light emission appropriate to some known $[Ca^{2+}]$—preferably the resting $[Ca^{2+}]$—and to assume that a square-law relation held between light and $[Ca^{2+}]$ above and below this level. It now appears that not only must the light emission from an aequorin-injected cell be determined for some known $[Ca^{2+}]$ but the shape of the relation between light and $[Ca^{2+}]$ must be determined under conditions appropriate to the intracellular milieu and over the range through which the intracellular $[Ca^{2+}]$ varies.

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Conjugal Transfer of the Gonococcal Penicillinase Plasmid

Abstract. *Certain gonococci, which heretofore have lacked a conjugal mating system, can sexually transfer a small plasmid (4.5 × 10⁶ daltons) which carries the gene for β-lactamase production. Frequencies of conjugal transfer were similar into diverse recipients (other gonococci,* Neisseria flava, *and* Escherichia coli*), which suggests that gonococci may transfer the plasmid promiscuously in nature.*

Recently, strains of penicillinase-producing *Neisseria gonorrhoeae* (PPNG) have been identified (*1*). The potential social and medical impact of these strains is considerable (*2*).

We have performed experiments demonstrating that penicillinase-producing gonococci can sexually transfer the penicillinase gene to other gonococci and to other species of bacteria. Up to the present, the only known mechanism of genetic transfer in the gonococcus has been by transformation (*3*). Past experiments that attempted to demonstrate conjugal transfer of drug resistance by gonococci were unsuccessful (*4*).

Table 1. Host range and transfer frequency of the gene for penicillinase production in penicillinase-producing gonococcal strain FA288. Strain FA306 is a spontaneous penicillinase-negative segregant of FA288. Strain FA305 is an unrelated strain derived from F62.

Penicillin-sensitive recipient	Frequency of penicillinase-producing transconjugants*
N. gonorrhoeae FA306	2×10^{-4}
N. gonorrhoeae FA305	6×10^{-6}
E. coli C600.5	2×10^{-4}
N. flava	1×10^{-5}

*Matings were generally for 16 hours in broth or on filters. Frequencies are expressed per number of donor CFU at onset of mating.

Two different naturally occurring PPNG isolates (FA288 and FA290) were tested for ability to act as conjugal donors of the penicillinase gene (*5*). Recipient strains included a spontaneous penicillinase-negative derivative of FA288, as well as other unrelated gonococci, and one isolate each of *Escherichia coli* (C600.5) and *Neisseria flava* (ATCC 14221).

Two different mating conditions were used. In the first, about 10^7 donor colony-forming units (CFU) per milliliter were mixed with the recipient (about 10^8 CFU/ml) in 10 ml of gonococcal base (GCB) broth, followed by incubation for 2 to 16 hours at 37°C with moderate agitation. Alternatively, equal amounts (about 10^8 CFU of each strain) were applied to sterile membrane filters (type HA; pore size, 0.45 μm, Millipore) and incubated overnight on GCB agar plates (*6, 7*). Deoxyribonuclease was included in the mating mixtures to inactivate any spontaneously released transforming DNA; concentrations were 100 μg on the filter and 50 μg/ml in broth. In all cases, the donor was a penicillinase-producing (or pencillin-resistant) strain, and the recipient was penicillin-sensitive. Selection for penicillinase-producing transconjugants was made on plates containing penicillin or ampicillin, and other antibiotics to inhibit the donor strain (*8*).

Cells were removed from the filters by agitation in sterile minimal medium (minimal medium Davis, Difco) and were generally washed twice by centrifugation before plating.

To diminish the effect of extracellular penicillinase, the plated samples were permitted to grow to visible colony formation (from 6 to 24 hours), at which time they were replica-plated to fresh antibiotic plates. Multiple serial replications were sometimes needed for identification of single transconjugants, particularly on plates containing $> 10^8$ cells from the mating mixture. Single colonies were purified and tested for penicillinase production (*9*), antibiotic resistance and nutritional requirements (*10*), and other characteristics.

Results (Table 1) showed that PPNG strain FA288 was able to donate the penicillinase gene to other gonococci, as well as to *E. coli* and *N. flava*. The other PPNG strain (FA290) did not donate its penicillinase gene in identical experiments. Frequency of transfer per input donor CFU of FA288 was approximately the same after either 16-hour mixed broth cultures, or on filters. Somewhat lower frequencies (1×10^{-5} to 1×10^{-6} per donor) were noted from 2-hour mixed broth cultures. Frequencies of transfer were slightly greater (twofold) when the donor culture was composed primarily of piliated clonal type 1 than nonpiliated type 4 cells (*11*).

Omission of deoxyribonuclease had no effect on transfer frequencies, suggesting that extracellular transforming DNA played little role in transfer of the penicillinase gene. Moreover, addition of DNA isolated from the various donor strains to the recipients under the same conditions, but in the absence of viable donor cells and without deoxyribonuclease, resulted in no penicillinase-producing colonies. Penicillinase-producing transformants were obtained at maximum frequencies of 1×10^{-7} under experimental conditions particularly suitable for transformation (*3*). Other experiments showed that transfer frequencies were identical into either piliated or nonpiliated gonococcal strains. Since transformation was several orders of magnitude more efficient into piliated than nonpiliated gonococci (*3*), we concluded that transfer of the penicillinase gene was not occurring by transformation.

Proof that conjugal transfer, rather than phage-mediated transduction, was occurring was provided by experiments showing that sterile filtrates of broth cultures of FA288 were unable to transfer penicillin resistance to any of the recipients.

Table 2. Relation between plasmid species and ability to produce penicillinase, and to serve as conjugal donor.

Strain	Origin	Phenotype		Plasmids* (10^6 daltons)
		Penicillinase	Conjugal donor	
N. gonorrhoeae				
FA288	Clinical isolate	Yes	Yes	2.5, 4.5, 25
FA293	Spontaneous derivative of FA288	No		2.5, 25
FA290	Clinical isolate	Yes	No†	2.5, 3.2
FA320	Spontaneous derivative of FA290	No		2.5
FA19	(*17*)	No		2.5
FA333	Transformant of FA19 from FA288	Yes	No†	2.5, 4.5
E. coli				
C600.5-1	Transconjugant from FA288	Yes	No†	4.5

*Plasmids were isolated by dye-buoyant density centrifugation (*18*) or by agarose gel electrophoresis (*19*). Approximate molecular size of plasmids from FA288 and FA290 were determined by electron microscopic measurement of contour lengths of open circular molecules as compared to SV40 (*18*). Molecular sizes of plasmids in other strains were determined on agarose gels (*19*). All gonococci contained the cryptic 2.5×10^6 dalton plasmid (*18*). †$< 3 \times 10^{-8}$ transconjugants per donor CFU after overnight Millipore filter mating.

Donor, recipient, and transconjugant strains were analyzed for the species of plasmids present. Results (Table 2) showed that the penicillinase gene in FA288 is carried on a plasmid of about 4.5×10^6 daltons; derivative strains that lost the ability to produce penicillinase lost this plasmid, and penicillinase-producing transconjugants (or transformants) from FA288 all acquired a plasmid of this size. The pencillinase plasmid of FA290 is slightly smaller than that of FA288.

In addition, the strain (FA288) which was able to serve as a conjugal donor also contains a larger plasmid of about 25×10^6 daltons, whereas FA290, which apparently cannot serve as a conjugal donor, lacks this plasmid. Transconjugants or transformants carrying the 4.5×10^6 dalton penicillinase plasmid from FA288 but not the 25×10^6 dalton plasmid (Table 2) were not able to conjugally transfer the penicillinase gene. It is possible, therefore, that conjugal transfer of the 4.5×10^6 penicillinase plasmid in FA288 is mediated by the 25×10^6 plasmid (*12*).

In some respects, these results are not surprising. It is well known that genes for penicillinase production in clinical isolates of the Enterobacteriaceae are usually carried on plasmids (*13*). Many plasmids that determine drug resistance (R factors) are associated with plasmids that promote conjugal transfer (resistance transfer factors) to the same or different species (*14*). Recently *Haemophilus influenzae*, which was not previously known to harbor drug-resistance plasmids, has also acquired a similar penicillinase plasmid (*15*), and it also can be conjugally transferred (*16*).

These results have several important implications. They clearly demonstrate that gonococci can transmit the penicillinase plasmid with relatively high efficiency to other gonococci, even after relatively brief matings. Transfer of the plasmid is considerably more efficient by conjugation than by transformation, which has heretofore been the only recognized mechanism of genetic exchange in the gonococcus. Conjugal transfer of plasmids between gonococci and other species raises the possibilities of future acquisition of other drug-resistance genes by recombination with other R plasmids, and of conjugal transfer of the penicillinase plasmid into other pathogenic *Neisseria*, such as the meningococcus. Although we did not perform experiments to test the latter hypothesis directly, it seems a reasonable possibility since FA288 donated as efficiently into *N. flava* as into *N. gonorrhoeae* or *E. coli* (Table 1).

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7. In the mixed broth experiments, donor and recipient strains were first grown to early log phase. In the filter experiments, cells were scraped off 16-hour plates.
8. In crosses between gonococci, the recipients were all constructed to be resistant to nalidixic acid (Nal^R), fusidic acid (Fus^R), and rifampin (Rif^R); the donors were sensitive to the same drugs. Selection for the penicillinase-producing

gonococcal transconjugants was made on plates containing penicillin at 0.2 to 1.0 μg/ml, rifampin at 5.0 μg/ml, fusidic acid at 1.0 μg/ml, and nalidixic acid at 2.0 μg/ml. Recipients of *N. flava* were Rif^R Nal^R, and selection was made on plates containing penicillin (1.0 μg/ml), rifampin (5 μg/ml), and nalidixic acid (2 μg/ml). *Escherichia coli* transconjugants were selected on ampicillin (30 μg/ml) plates.

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Conditioned Narcotic Withdrawal in Humans

Abstract. *Subjective and physiological manifestations of the narcotic withdrawal syndrome were produced as a conditioned response. Withdrawal reactions precipitated by the narcotic antagonist naloxone in methadone-dependent volunteers were the unconditioned response. These data support clinical anecdotes of withdrawal symptoms occurring in former addicts when they return to their drug-related environment.*

Resumption of narcotic use is a serious problem among treated addicts. Clinical evidence suggests that conditioning factors may play a role in relapse (*1*). Former addicts have reported the return of withdrawal symptoms when they visit areas of drug use (*2*). Wikler first proposed a conditioning explanation for these reports (*3*), and subsequent animal research (*4*) has provided support. Recently we reported an experimental method for producing conditioned withdrawal in human subjects (*5*). We now report objective evidence of conditioned narcotic withdrawal experimentally produced in eight male former heroin addicts.

The subjects were maintained on a constant daily dose of methadone, with daily urine test evidence confirming their lack of street drug use. Their median age was 26 years (mean, 26 years; range, 22 to 29 years), median education was 12 years (mean, 12 years; range 9 to 16 years), and median duration of addiction was 7 years (mean, 7 years; range, 4 to 10 years). Five subjects were white and three were black. There were two controls, one white and the other black. All volunteered for the study and gave their written informed consent after a detailed explanation of the procedure. The events that would take place during a session were described to participants; they were also informed that they would receive either saline or naloxone, a short-acting narcotic antagonist, which would cause mild withdrawal symptoms. Intramuscular injections of naloxone (0.1 mg) were the unconditioned stimulus. These injections caused the onset of tearing, rhinorrhea, yawning, decreased skin temperature, increased respiratory rate, and increased heart rate; these reactions lasted about 20 to 30 minutes and were conditioned to environmental cues. Methadone dosage ranged from 25 to 70 mg (mean, 43 mg; median, 40 mg). By using a constant dose of naloxone, the unconditioned withdrawal reactions were kept at approximately the same magnitude within and among participants (*6*).

Participants were tested individually in 1-hour sessions in a sound-attenuating chamber where heart rate, skin temperature, respiration, motor responses, and subjective reports were monitored and recorded. Introductory instructions were presented each day at the beginning of the session. At this time the patient had certain procedures explained to him (such as where he should place his head for pupil measurements and how he should answer questions about severity of withdrawal symptoms). After instructions, the participant heard background music for a 10-minute period of baseline measurement. Naloxone was then administered by a nurse using a standard procedure. The background music gradually became softer as a tone and odor (compound conditioning stimulus) (CS) became stronger. The tone (700-hertz, audiogenerator) was tape-recorded and was therefore the same for all subjects. It began at a low level 3 minutes after injection, became audible 7 to 8 minutes after injection, reached peak intensity (70 db) at 13 minutes, and remained at that intensity until 18 minutes, when it decreased at the same rate and became inaudible again 28 minutes after injection. The odor was administered by passing compressed air through a flask that contained gauze soaked in oil of peppermint. Both components of the CS complex increased and decreased at the same rate. This timing of the CS was selected because it corresponded to the pattern of unconditioned withdrawal as determined in pilot studies. As the withdrawal subsided, the tone and odor were replaced by music different from the first. At this time the participant received his daily dose of methadone. Physiological and psychological monitoring was conducted continuously throughout the session; the session ended 20 minutes after ingestion of methadone, which corresponded with termination of withdrawal.

Each subject received 21 sessions, five per week. The first three sessions consisted of normal saline baseline trials. Three consecutive conditioning sessions were then given, followed by a single test session. This sequence of three conditioning sessions and one test session was presented four times (total of 12 conditioning sessions); the last block of conditioning trials was followed by three test trials rather than one. This sequence and number of sessions was chosen as the maximum that outpatient volunteers could be expected to complete. Test trials were arbitrarily included every fourth session to obtain data on acquisition of conditioning. The two controls were treated identically to the eight subjects, except that they received only saline injections during all sessions.

Evidence of conditioning was obtained by comparing trials 2 and 3 of the baseline block (saline trials, before conditioning) with trials 19 and 20 (saline trials, after conditioning). The first baseline trial was omitted from the analysis because many participants were anxious and anticipated severe withdrawal reactions. The last test trial was also omitted because any conditioned response that had developed began to be extinguished by the third consecutive nonreinforced trial. The degree of conditioning was assessed by using a t-test for paired observations in which each subject's baseline scores were compared to his test trial scores. When data for all eight conditioning subjects were pooled, respiration rate showed a significant increase on the test trials ($t = 2.50$, d.f. $= 7$, $P < .025$) and skin temperature showed a significant decrease ($t = 1.92$, d.f. $= 7$,