tious unit is lipid solvent-sensitive and acid labile ($pH \leq 5$), and that a very small proportion ($\leq 10^{-6}$) of the agent population will pass a membrane filter of 100-nm pore size (*1*). Curves showing growth of the agent after inoculation of A-549 cells with large numbers of infectious units reveal an eclipse phase of 6 to 8 hours in which less than 10^{-4} of the inoculum is recoverable, an observation that lends additional support to the long-standing hypothesis (*3*) that the etiologic agent of KHF is a virus.

Attempts to isolate the agent from humans and suspected rodent reservoirs in various geographic regions of the world have revealed that A-549 cells are not uniformly susceptible to all strains of the agent or related agents. On the Korean peninsula, for example, native *A. a. coreae* remains the host of choice for initial isolation of the agent. However, replication of KHF strain 76-118 in A-549 cells makes it possible to study the KHF agent in vitro and provides a means of diagnosing hemorrhagic fevers with renal syndrome in laboratories outside Korea. Although *A. a. coreae* is an adequate host for these purposes, the rodent has not been colonized and must be captured and returned to the laboratory from KHF-free areas of the Korean peninsula.

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12. Tissue culture cluster-24, No. 3524, COSTAR, Cambridge, Mass.
13. Previous investigators have used other continuous and primary cell cultures, including dog embryo (R-1247), porcine kidney (PS), mink lung (MV1Lu), Chinese hamster lung (Dede), primary human embryonic lung, and primary rat liver (*1*); HEp-2, HeLa, FL, Detroit, six lines of human origin, and primary Korean hamster heart and kidney (*8*); rhesus monkey kidney (MA-104), newborn rabbit kidney (MA-111), primary hamster kidney (*5*), and rhesus monkey kidney (*1, 5, 8*).
14. Supported in part by grants DAMD17-77-G-9431 and DAMD17-78-G9444 of the U.S. Army Medical Research and Development Command, Fort Detrick, Frederick, Md. 21701. In conducting the research described herein, the U.S. Army investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council.
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Receptor for Albumin on the Liver Cell Surface May Mediate Uptake of Fatty Acids and Other Albumin-Bound Substances

Abstract. *Kinetic analysis of the uptake of carbon-14-labeled oleate in a single-pass perfusion of rat liver and saturable and specific binding of iodine-125-labeled albumin to hepatocytes in suspension suggest the existence of a receptor for albumin on the liver cell surface. The putative receptor appears to mediate uptake of albumin-bound fatty acids by the cell and may account for the efficient hepatic extraction of many other substances tightly bound to albumin.*

Long-chain fatty acids and many other substances, although tightly bound to albumin, are efficiently removed from plasma during passage through the liver. Uptake of these substances has been believed to follow their spontaneous dissociation from albumin in the bulk aqueous phase, with subsequent diffusion of free ligand to the surface of the liver cell. We now present evidence that uptake of long-chain fatty acids takes place by saturable interaction of the fatty acid–albumin complex with a receptor for albumin on the liver cell surface (*1, 2*).

Materials used in this study included oleic acid (Calbiochem), [1-^{14}C]oleic acid and Na^{125}I (New England Nuclear), bovine serum albumin (fraction V, essentially fatty acid–free), bovine γ-globulin (fraction II), human transferrin, rat serum albumin (fraction V), ovine prolactin, and ovalbumin (Sigma), and Fluosol-43 fluorocarbon emulsion (Alpha Therapeutics). Albumin, after further purification by ion-exchange chromatography, was iodinated by the chloramine-T method to a molar ratio of ~ 1.0 (*3*). Livers were from female Sprague-Dawley rats (Simonsen), aged 50 to 55 days, given free access to a standard laboratory diet. Each liver was perfused for 30 minutes with recirculating oxygenated fluorocarbon in Krebs-Henseleit buffer (Fluosol-43) containing 0.2 percent glucose (weight to volume); this was followed by a 4-minute single-pass perfusion with buffer and glucose alone to remove fluorocarbon from the system. The liver was then perfused (single pass) with a sequence of up to seven solutions containing various concentrations of albumin and [^{14}C]oleate in buffer at a flow rate of 3.8 ± 0.2 ml/min per gram of liver [mean ± standard error (S.E.)]. Steady-state uptake, achieved within 1 minute, was determined at each substrate concentration. Viability of the liver was assessed by electron microscopy, oxygen consumption, bile flow, and release of cellular enzyme markers (*4*). Fatty acids were extracted from effluent samples (*5*), and 1-ml portions of the organic phase were assayed in duplicate for radioactivity. The amount of [^{14}C]oleate esters in the effluent was insignificant for up to 15 minutes after the [^{14}C]oleate perfusion was started (*6*), but where appropriate, measurements were corrected for the presence of ^{14}C-labeled lipids other than fatty acids. No free fatty acids other than oleate were detected in the effluent by gas-liquid chromatography. Net uptake per gram of liver was calculated as the product of the steady-state drop in the [^{14}C]oleate concentration across the liver and the flow rate per gram of liver

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(albumin concentration was unaffected). Binding of ^{125}I-labeled bovine albumin to hepatocytes in suspension was determined by incubating cells (~ 10 mg of cell protein per milliliter) (*7*) with ^{125}I-albumin (1.5×10^{-7} to 4.5×10^{-4} *M*) at 20°C for 30 minutes. Cell-associated ^{125}I was measured after centrifugation and removal of the supernatant.

In perfused liver, when [^{14}C]oleate concentration was increased (0.05 to 0.5 m*M*) while albumin concentration was fixed (0.15 m*M*), oleate uptake was linearly related to oleate concentration and showed no evidence of saturation (Fig. 1a). In contrast, when the concentrations of albumin and oleate (fixed at a molar ratio of 1:1) were increased in parallel, the uptake of oleate suggested a saturable process (Fig. 1b). This apparent saturation did not reflect changes in function or viability of the liver, since identical results were obtained when the perfusate solutions were administered in reverse order. Furthermore, in neither of the above experiments was uptake correlated with changes in the calculated equilibrium concentrations of unbound oleate (Fig. 1, a and b) (*8*).

A double-reciprocal plot of the data was linear except at very low substrate concentrations, where uptake velocity was slightly greater than expected (Fig. c). This result suggested that at higher (that is, physiological) concentrations, corresponding to the linear portion, uptake predominantly reflected a saturable process in which the oleate-albumin complex was substrate; nonlinearity at very low concentrations apparently was due to a second uptake process evident only when the concentration of oleate-albumin complexes was low.

Computerized curve fitting was used to test this and other possible interpretations of the findings. In all experiments, an excellent fit with the data (Fig. 1c) was obtained with a model consisting of two uptake components: (i) a major saturable component, the substrate for which is oleate-albumin complex, and (ii) a minor invariant component. Models in which there was no saturable uptake component responding to the oleate-albumin concentration or in which unbound oleate was a major uptake determinant were not compatible with our data (*9*).

Kinetic constants were derived from data obtained by perfusing livers from six female rats. The apparent values of the Michaelis constant (K_m) and the maximum velocity (V_{max}) for the saturable process, calculated for each liver after correction for the constant minor uptake component (3.8 ± 1.0 nmole/min per gram of liver), were 0.198 ± 0.022 m*M* and 380 ± 40 nmole/min per gram of liver, respectively (*10*).

In order to test the possibility that the putative receptor also might recognize uncomplexed albumin, we performed the kinetic analysis under identical conditions except that each albumin concentration was increased by 66 μM. Under these conditions, the apparent V_{max} was unchanged (351 ± 77 nmole/min per gram of liver) in comparison with controls, whereas the apparent K_m was increased by 43 percent (0.283 ± 0.022 m*M*; $P < .02$) (Fig. 1d). The apparent inhibition constant (K_I) for albumin is 0.154 ± 0.050 m*M*.

These data support a two-component model for the uptake of long-chain fatty acids by the perfused rat liver. The major component, accounting for more than 95 percent of uptake, exhibits saturation kinetics only when the concentrations of albumin and oleate are varied in parallel, and not when oleate alone is varied. Furthermore, albumin appears to inhibit the saturable process competitively.

Data on binding of labeled albumin to hepatocytes was evaluated with a nonlinear least-squares computerized curve-fitting program. The results (Fig. 2) were compatible with the existence of a single high-affinity binding site [the dissociation constant (K_d) was 25 ± 7 μM, (mean ± S.E.), with (10 ± 3) × 10^6 sites per cell ($N = 4$)] plus low-affinity, high-capacity binding that was not clearly saturable. Bound ^{125}I-albumin (0.15 μM) was not displaced from hepatocytes by ovalbumin, bovine gamma globulin (*11*), human transferrin, or sheep prolactin (0.15 m*M*). Displacement by rat albumin was comparable to that by unlabeled bovine albumin. This indicates that binding was both saturable and specific (*2*, *12*), and that the affinities of rat and bovine albumin are similar.

Our findings suggest that the major uptake component is attributable to a receptor for albumin, on the liver cell sur-

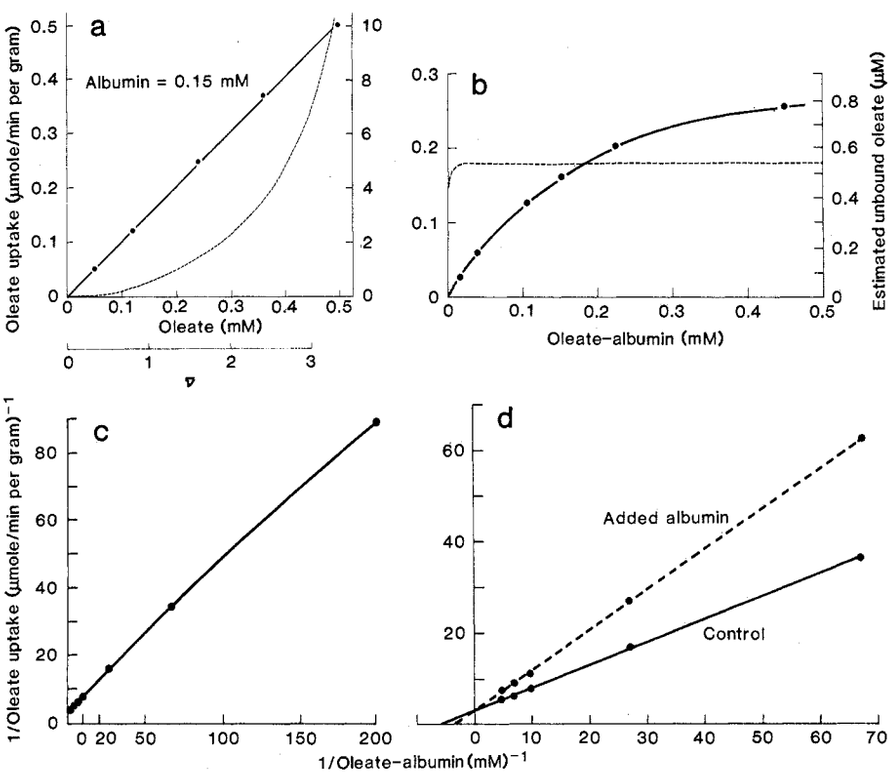

Fig. 1. Oleate uptake by representative isolated perfused rat livers. Livers were perfused (single pass) with a sequence of solutions containing the indicated concentrations of [^{14}C]oleate and bovine serum albumin. Steady-state oleate uptake was calculated by analysis of effluent samples. (a) Effect of varying oleate concentration on (—) uptake and (---) unbound oleate concentration, with albumin fixed at 0.15 m*M*. (b) Effect of varying the concentration of both oleate and albumin at a 1:1 molar ratio on (—) oleate uptake and (---) unbound oleate concentration. (c) Double-reciprocal plot of [^{14}C]oleate uptake as a function of oleate and albumin concentrations (1:1 molar ratio). The line is a nonlinear least-squares computer fit based on a model for uptake consisting of a major saturable component with the oleate-albumin complex as substrate, and a minor constant component (see text). (d) Double-reciprocal plot of [^{14}C]oleate uptake as a function of oleate and albumin concentrations (1:1 molar ratio) (---) with and (—) without added uncomplexed albumin (66 μM). The data indicate saturation kinetics and competitive inhibition by the uncomplexed albumin. Uptake has been corrected for the minor uptake component.

face, that catalyzes the uptake of albumin-bound oleic acid. This model may be depicted as follows

$$\begin{array}{c} [A{:}FA] + R \leftrightharpoons [A{:}FA]{:}R \rightleftharpoons \\ FA \quad + \quad A{:}R \\ \downarrow \qquad\quad \Uparrow\Downarrow \\ \text{Uptake} \quad A + R \end{array}$$

where A represents uncomplexed albumin; FA, unbound oleate; R, the albumin receptor; [A:FA], the albumin-oleate complexes; and [A:FA]:R and A:R, the albumin receptor bound to complexed and uncomplexed albumin, respectively. The rate of the minor uptake component appeared to be constant, despite a major change in the equimolar concentrations of albumin and oleate. Since the concentration of unbound oleate was also nearly constant under these conditions (*8*), it is likely that the minor component reflects uptake of unbound oleate. The absence of a detectable minor uptake component (by either computer or graphic analysis) in the albumin competition studies (Fig. 1d) is consistent with this interpretation, since the unbound oleate concentration would be greatly reduced by the addition of albumin (*13*). The model proposed for the minor component is

$$\begin{array}{c} [A{:}FA] \leftrightharpoons FA + A \\ \downarrow \\ \text{Uptake} \end{array}$$

In interpreting these findings, it is important to recognize that in an equimolar solution of albumin and oleate, complexes other than the 1:1 are present in significant concentrations (*14*). Nevertheless, the linear response of oleate uptake to increasing oleate and fixed albumin concentrations (Fig. 1a) indicates that the interaction of albumin with the putative albumin receptor is not influenced substantially by the number of fatty acid molecules bound to the individual albumin molecules. Thus, as the number of fatty acid molecules bound to a given albumin molecule increases, there is a proportional increase in the amount of fatty acid available for uptake when that albumin molecule binds to the receptor (Fig. 1a). Saturation occurs only under conditions in which the availability of albumin receptors becomes limiting (Fig. 1b).

Our data exclude both unbound and total fatty acid concentrations as principal determinants of uptake. Unbound fatty acid is excluded as the critical determinant by the lack of correlation between uptake and unbound oleate concentrations (Fig. 1, a and b). Total oleate concentration (bound plus unbound) is excluded as the major uptake determinant by the data in Fig. 1d in which

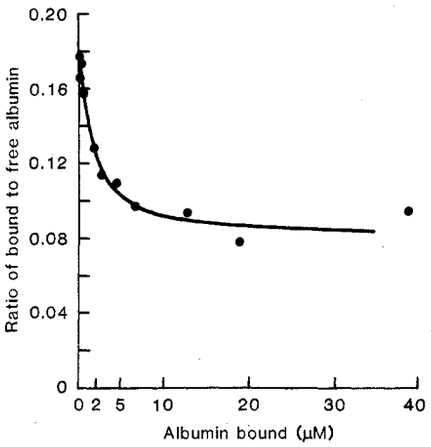

Fig. 2. Scatchard plot of binding of ^{125}I-albumin to isolated hepatocytes. The line is a nonlinear least-squares computer fit based on a binding model consisting of a single class of relatively high-affinity binding sites and a second binding component that is not clearly saturable.

oleate uptake is diminished by the addition of albumin. Thus, the data directly support the concept that the concentrations of albumin and albumin-bound oleate are the principal determinants of uptake, and they exclude the major alternative possibilities.

The existence of a hepatocyte receptor for albumin is also supported on theoretical grounds. Under physiological conditions, only about 0.1 percent of long-chain fatty acids are not bound to albumin (*8*). If this small fraction alone were to account for uptake, its regeneration from the oleate-albumin complex would need to be extremely rapid to permit the 20 to 40 percent overall extraction observed during a single pass through the liver (*6*). However, the half-time for the dissociation of long-chain fatty acids from high-affinity sites on albumin is significantly longer than the time required for blood to pass through the liver (*15*). Not only is this spontaneous dissociation relatively slow, but it is unlikely to lead to uptake, since most of the newly dissociated fatty acid would reassociate with albumin in the bulk aqueous phase before it could diffuse to the liver surface. Thus, a mechanism by which the probability of uptake of fatty acid can be increased would seem essential; the putative albumin receptor appears to provide this mechanism.

Similar reasoning can be applied to the uptake of other ligands such as sulfobromophthalein and bilirubin, which are bound to albumin more tightly than oleate and are even slower to dissociate (*15, 16*). Recent data have shown uptake kinetics for these two organic anions which are similar to those described for oleate (*17*). Thus, the putative hepatic albumin receptor may participate in the uptake of a wide variety of other endogenous and exogenous albumin-bound substances, including many bile acids, hormones, drugs, toxins, and carcinogens.

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9. Models considered with the relative standard errors of their respective computed regression lines: single saturable site plus constant (the preferred model), 1.00; single saturable site plus unsaturable process, 2.50; and single saturable site alone, 2.72. An additional alternative model, not excluded by the data, has two sites, the second of which has a very high affinity and low capacity, such that its contribution to uptake is essentially constant under the conditions studied.
10. The observed V_{max} applies only to a fixed molar ratio of oleate to albumin of 1:1. Oleate uptake rates exceeding the V_{max} can be obtained at higher molar ratios (Fig. 1a, solid line). The observed K_m should be viewed as a rough approximation of the actual physiological value for several reasons; (i) bovine rather than rat albumin was used. (ii) The flow rates used in the perfusion studies and the absence of erythrocytes may change the observed K_m by altering diffusion barriers adjacent to the cell (*12*). (iii) The uptake of oleate as the perfusate passes through the liver should generate an oleate concentration gradient within the lobule. Alternative methods for calculating these kinetic constants, in which substrate (oleate-albumin) concentration was taken as equal to that in the effluent, or to the mean of perfusate and effluent, gave qualitatively similar interpretations and only minor differences in the derived values.
11. A slight displacement by bovine γ-globulin was seen consistent with its known contamination by albumin (~ 1 percent).
12. The K_d for albumin binding by hepatocytes at equilibrium is one-eighth the apparent K_m for the oleate-albumin complexes at steady state. This may reflect depletion of oleate and accumulation of inhibiting uncomplexed albumin at the surface of the cell because of diffusion barriers (for example, unstirred water layer, space of Disse) in the uptake studies [J. M. Dietschy in *Disturbances in Lipid and Lipoprotein Metabolism*, J. M. Dietschy, A. M. Gotto, Jr., J. A. Ontko, Eds. (Waverly, Baltimore, 1978), pp. 1-28].
13. Direct measurement of the uptake of unbound oleate (0.05 to 0.5 μM) from albumin-free solutions gave rates of 4 ± 2 mmole/min per gram of liver per mole/liter, while the rate due to the minor uptake component divided by the calculated

unbound oleate concentration was 7.6 ± 2.0. While this agreement is further evidence that the minor component is due to unbound oleate, these results should be interpreted with caution since uptake of the albumin-free oleate may have been flow-limited (single-pass extraction averaged 90 percent).

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3 September 1980

Anabaseine: Venom Alkaloid of *Aphaenogaster* Ants

Abstract. *Anabaseine, a tobacco alkaloid, is identified as a poison gland product in* Aphaenogaster *ants, in which it functions as an attractant.*

Collecting *Aphaenogaster* ants by mouth-operated insect aspirator produces a brief burning sensation in the mouth and throat similar to that felt during aspiration of the myrmicine ant *Monomorium pharaonis*. Because dialkylpiperidines, pyrrolidines (*1*), dialkylpyrrolidines (*2*), and an indolizine (*3*) have been found in the poison glands of myrmicine ants, we decided to analyze *Aphaenogaster* species for similar alkaloids. Here we report that the poison glands of *Aphaenogaster fulva* and *A. tennesseensis* (*4*) contain the tobacco alkaloid anabaseine (Fig. 1). We also show that anabaseine serves as an attractant in *A. fulva*.

Methylene chloride extracts of whole ants and abdomens were analyzed by combined gas chromatography–mass spectrometry (*5*). In addition to the usual alkanes and alkenes from Dufour's gland, a major peak was noted which exhibited a molecular ion at mass-to-charge ratio (*m/e*) 160, with additional fragments at *m/e* 159, 156, 145, 131, 104 (base peak), 78, and 51. Extracts of excised poison glands showed that this peak represented the predominant volatile (> 90 percent). The compound was established as a base by its solubility in 5 percent hydrochloric acid, which allowed separation from the hydrocarbons. A proton magnetic resonance spectrum (*6*) of the basic material revealed the following: a chemical shift (δ) of 8.95, singlet, 1 H; 8.6, broad singlet, 1 H; 8.1, doublet, 1 H; 7.3, doublet, 1 H; 3.85, triplet, 2 H; 2.62, triplet, 2 H; 1.85, multiplet, 2 H; and 1.70, multiplet, 2 H. These data are consistent with a 3-substituted pyridine (*7*) in which the substituent has four nonequivalent methylene units. Deuterium oxide exchange indicated that only one of these methylene units was labile, as evidenced by the disappearance of the peak at δ 2.62.

Treatment of the natural produce with chloranil (*8*) gave an aromatic product identical to 2,3′-bipyridyl (*9*). Anabaseine was synthesized by the method of Spath and Mamoli (*10*), giving material whose mass spectrum, proton magnetic resonance spectrum (*11*), and gas chromatographic retention time (isothermal coelution) were identical to those of the natural product. The unusual loss of methyl and ethyl groups (*m/e* 145 and 131) in the mass spectrometer requires rearrangement, but this process is known in tobacco alkaloids (*12*). The peak at *m/e* 156 appears to show dehydrogenation to 2,3′-bipyridyl in the mass spectrometer. Proton magnetic resonance spectra of extracts of dissected poison glands and gas chromatographic comparison with standard solutions indicated that the amount of anabaseine present in each ant is ≤ 1 μg.

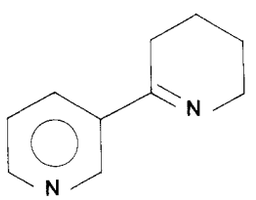

Fig. 1. Structure of anabaseine.

Although anabaseine is a minor alkaloid in tobacco (*12*) and has been identified as a toxin in a nematode (*13*), it has not previously been found in insects. In an effort to establish the chemobehavioral function of anabaseine in *Aphaenogaster* ants, we placed anabaseine-treated paper disks (*14*) in the foraging arena of a laboratory colony of *A. fulva*. Within minutes, six to eight ants appeared in the vicinity of each treated disk. In repeated trials, these disks continued to be attractive for 8 to 10 minutes. Although control disks elicited no noticeable reaction, worker ants approached the treated disks with their heads raised, mandibles open, and antennae outstretched at a 45° angle. They picked up some of the treated disks and carried them into the colony or around the foraging arena. When control and treated disks were placed in a colony of carpenter ants (*Camponotus pennsylvanicus*), both were ignored. Since *Aphaenogaster* and *Camponotus* feed on similar foods, the rejection by *C. pennsylvanicus* and acceptance by *A. fulva* of treated disks indicates that they were reacting to a pheromone and not exhibiting a gustatory response.

To measure the attractant response, we placed control and treated disks in the center of separate 8-cm-diameter circles drawn in the foraging arena of a laboratory colony of *A. fulva*. Ants entered the experimental circles twice as often as the control circles over repeated 15-minute periods.

The data in Table 1 confirms that anabaseine does not elicit a gustatory response and is still an attractant. Mealworm (*Tenebrio molitor*) homogenates (*15*) can serve as food for *A. fulva* and are attractive. Homogenates containing

Table 1. Reactions of *A. fulva* workers to mealworm homogenates containing anabaseine. We added 5 μl of methylene chloride or 5 μl of 0.5 percent anabaseine to 10 μl of mealworm homogenates and placed each mixture in the foraging arena of *A. fulva* colonies. The ants were observed for 5- and 10-minute intervals and their feeding behavior was scored as repelled or attracted. In repelled behavior the ant, after contacting the test solution with its mouthparts, fed for less than 5 seconds. This behavior was frequently followed by the ant backing up very quickly and pausing to clean its mouthparts and antennas. In attracted behavior the ant, after touching the solution with its mouthparts, fed for 5 seconds or longer.

Treatment	Test series 1			Test series 2		
	0 to 5 minutes observation	5 to 10 minutes observation	Total	0 to 5 minutes observation	5 to 10 minutes observation	Total
Anabaseine						
Repelled	16	28	44	15	20	35
Attracted	2	3	5	3	5	8
Control						
Repelled	1	3	4	2	1	3
Attracted	12	14	26	10	15	25

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