Table 1. Radioactive binding to bentonite in solutions of [^{3}H]leucine and [^{3}H]aspartic acid. Bentonite samples were shaken with the amino acids in 50 mM tris-HCl buffer at 30°C for 15 minutes (leucine) or 90 minutes (aspartic acid). The supernatant obtained on the centrifugation of bentonite sample 1 was each time used to measure binding with sample 2. The bentonite was washed rapidly and counted as described in the text.

Tritiated amino acid	Bentonite sample	Concentration ($\times 10^{-7}M$)	Available counts (%)	Concentration (picomoles per 10 mg of bentonite)
L-Leucine	1	3.6	0.043	0.15
L-Leucine	2	3.3	0.03	0.10
DL-Leucine	1	3.1	0.045	0.14
DL-Leucine	2	3.3	0.025	0.08
L-Aspartic acid	1	6.6	0.21	1.2
L-Aspartic acid	2	4.6	0.18	0.87
D-Aspartic acid	1	6.6	0.26	1.7
D-Aspartic acid	2	4.6	0.17	0.82

small to be measured in terms of a change in the supernatant solution. We measured the binding of radioactivity by counting the washed bentonite. The washing time was reduced as far as possible to minimize the reverse process. We were able to complete three washes in ice-cold buffer in 12 minutes by braking the centrifuge rapidly after each 1-minute centrifugation. The adequacy of this procedure was established by the fact that the third wash liquid had a count approximating that of the normal background. The samples of washed bentonite were counted in the same way as in the earlier study (*1*).

Using the procedures outlined above, we found that the binding to bentonite with $10^{-8}M$ amino acids was too low for accurate measurement. The amino acids were therefore used at concentrations of 2×10^{-7} to $6 \times 10^{-7}M$, and bentonite was used at 5 to 10 mg/cm^3 instead of 2 mg/cm^3. The maximum binding of [^{3}H]leucine was reached in 15 minutes as described in (*1*), but the maximum binding of [^{3}H]aspartic acid required an exposure of 90 minutes at 30°C.

The radioactive supernatants from the bentonite were recovered, and fresh samples of bentonite were incubated for the appropriate time. If the counts bound the first time were due to the amino acids, the removal of a small percentage of the available counts should not have reduced the counts bound to the second sample of bentonite by more than this small percentage. Table 1 shows data for the binding of leucine and aspartic acid to two successive samples of bentonite. With aspartic acid the second concentration was lower.

The concentrations used in these experiments were approximately 20 times those used by Bondy and Harrington (*1*). The levels of binding are nevertheless lower than the 26.8 and 4.1 pmole per 10 mg for L- and D-leucine (*1*). No preferential binding of the L isomer is apparent in our data. The higher binding of D-aspartic acid to the first sample of bentonite is not observed with the second sample; this result suggests a higher degree of contamination of the tritiated D amino acid. The decline in the leucine bound to the second sample of bentonite confirms the binding of material that is not leucine, since for a decline in 0.04 percent of the counts there has been a decline in bound counts of about 45 percent. This was further confirmed by the observation that bentonite bound more counts when [^{3}H]leucine had been heated. After 50 minutes at 100°C, the percentage of counts bound increased from 0.02 percent of a freshly prepared solution to 0.11 percent for $3.3 \times 10^{-7}M$ leucine. In all the experiments that we carried out the percentages of counts bound were within the known limits of impurity.

Bondy and Harrington (*1*) showed a reduction in the binding of ^{3}H-labeled L amino acids in the presence of excess unlabeled L but not D amino acids. We did not observe this result in our study where the addition of $10^{-2}M$ unlabeled D- or L-leucine to $3 \times 10^{-7}M$ labeled DL-leucine only reduced the binding from 0.07 to 0.06 percent with added L-leucine and to 0.05 percent with added D-leucine. Bentonite labeled with [^{3}H]leucine was washed and suspended in buffer or in buffer with $10^{-2}M$ of the L or D isomer. The same release of counts was observed under the three conditions.

Similarly, the addition of $10^{-2}M$ of either D or L unlabeled aspartic acid reduced the binding of $4.6 \times 10^{-7}M$ L-[^{3}H]aspartic acid from 0.14 to 0.02 percent of the available counts.

Thus no selective binding of L amino acids by samples of sterile bentonite from four sources was observed. The observed binding is largely attributed to the products of radiochemical decomposition because (i) storage and heating of the dilute solutions increased the percentage of counts bound, (ii) the counts bound were always below the known levels of impurity of the radiochemicals, and (iii) a second addition of bentonite bound 45 percent less leucine after a loss of only 0.04 percent of the total counts.

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4. Technical assistance by J. Murray was funded by a Monash University research grant. S. C. Bondy's responses to our questions were much appreciated.

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Cultivation in vitro of the Vivax-Type Malaria Parasite *Plasmodium cynomolgi*

Abstract. *The vivax-type simian malaria parasite* Plasmodium cynomologi *was cultured in vitro by both the candle jar method and the continuous flow technique, with rhesus monkey erythrocytes and RPMI 1640 medium supplemented with Hepes buffer and human serum. After 6 weeks in culture, the growth of the parasite had permitted a* 5×10^6 *cumulative dilution of the original inoculum. Cultured parasites remained infective to rhesus monkeys and exhibited a reversible decrease in the ameboid behavior of their trophozoites.*

Plasmodium vivax contributes substantially to the negative socioeconomic impact of malaria in the developing world (*1*). This human malaria parasite is widely distributed and causes a recurrent and debilitating disease. *Plasmodium cynomolgi*, the simian counterpart of *P. vivax*, is often used in research directed at *P. vivax*. We now report the successful continuous cultivation of *P. cynomolgi*, the first vivax-type malaria parasite to become accessible to long-term studies in vitro.

Plasmodium cynomolgi occurs in Old

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World monkeys (*2*). Experimental infections in the rhesus monkey, *Macaca mulatta*, with *P. cynomolgi* are very similar to human infections with *P. vivax* in that both follow a generally nonfatal, chronic course (*3*). The blood stages of both species show a tertian (48-hour) periodicity in their asexual cycle and are essentially similar in their morphology and in the changes that they induce in the host erythrocyte (*4*). Sporozoite-induced infections of the appropriate host with these two parasites are characterized by true relapses (*3*) resulting from the reactivation of dormant liver stages (*5*).

We cultured *P. cynomolgi* using a modification of the candle jar method (*6*). To initiate the first culture line, heparinized blood containing 44,000 parasites per cubic millimeter was collected from a rhesus monkey experimentally infected with *P. cynomolgi* (Berok strain) (*7*). The parasitized blood was washed and the erythrocytes were resuspended to 8 percent in a culture medium consisting of RPMI 1640 medium (Gibco) (*8*) supplemented with 30 m*M* Hepes buffer and 10 percent human O+ serum (*9*). This culture material was distributed in 0.5-ml amounts into 16-mm flat-bottomed wells (Disposo-Trays, Linbro) and incubated in a candle jar at 37°C. The overlying spent medium was removed and replaced with fresh medium daily. At 3- to 5-day intervals, subcultures were made by rediluting four- to tenfold the parasites with fresh, uninfected rhesus monkey erythrocytes (*10*). Giemsa-stained thin smears were used for counting the parasites.

This first culture line was initiated at a parasite count of 151 parasites per 10^4 erythrocytes. Two days later, after the first cycle in vitro, the count had doubled; it reached 569 by day 5, when the first subculture was made (Table 1). During the following weeks, parasite counts of 300 to 400 per 10^4 erythrocytes were routinely obtainable, with peaks of up to 1000 occasionally observed. Four- to tenfold increases in parasite counts over 3- to 5-day periods compensated easily for the parasite dilutions resulting from subcultures. This has permitted to date a 5×10^6 cumulative dilution of the original inoculum after 42 days in vitro.

We initiated three additional culture lines with equal success, using blood collected from rhesus monkeys experimentally infected with the same strain of *P. cynomolgi*. In two lines where the inoculum consisted mainly of ring stages, the parasites matured synchronously into schizonts and a new generation of rings over a 2-day period, thus confirming in vitro the tertian periodicity of *P. cynomolgi*.

We also successfully cultured *P. cynomolgi* by the continuous flow technique (*11, 12*). A culture line previously grown by the candle jar method, upon transfer into a flow vessel, maintained a consistently good growth pattern. This technique has permitted to date a 3×10^5 cumulative dilution of the flow vessel inoculum after 34 days in culture.

Cultured *P. cynomolgi* remained infective to monkeys. Parasites grown in vitro for 13 and 21 days were inoculated intravenously into two splenectomized rhesus monkeys. One day after inoculation, parasites were observed in the peripheral blood and both animals developed the nonlethal, chronic infection typical of *P. cynomolgi* in this host.

In culture, *P. cynomolgi* retained most of its normal morphological characteristics. Infected erythrocytes were enlarged and presented the typical Schüffner's stippling. Multiple infections were occasionally observed (Fig. 1A). Mature schizonts generally had 10 to 20 merozoites (Fig. 1E); and gametocytes, although

Table 1. Number of *P. cynomolgi* (Berok strain) during the first 4 weeks in continuous culture by the candle jar method.

Day of culture	Cumulative dilution	Parasites per 10^4 erythrocytes				
		Rings	Trophozoites	Schizonts		Total
				Two nuclei	More than two nuclei	
0		91	58	1	1	151
2		76	181	18	40	315
5		328	191	9	41	569
5	5	58	48	0	4	110
9		105	237	40	37	419
9	25	32	28	5	13	78
13		198	251	11	17	477
13	125	33	56	1	4	94
17		226	99	17	48	390
17	1000	26	17	2	5	50
19		47	43	9	16	115
22		209	200	22	52	483
22	6000	43	18	0	6	67
27		202	137	22	23	384

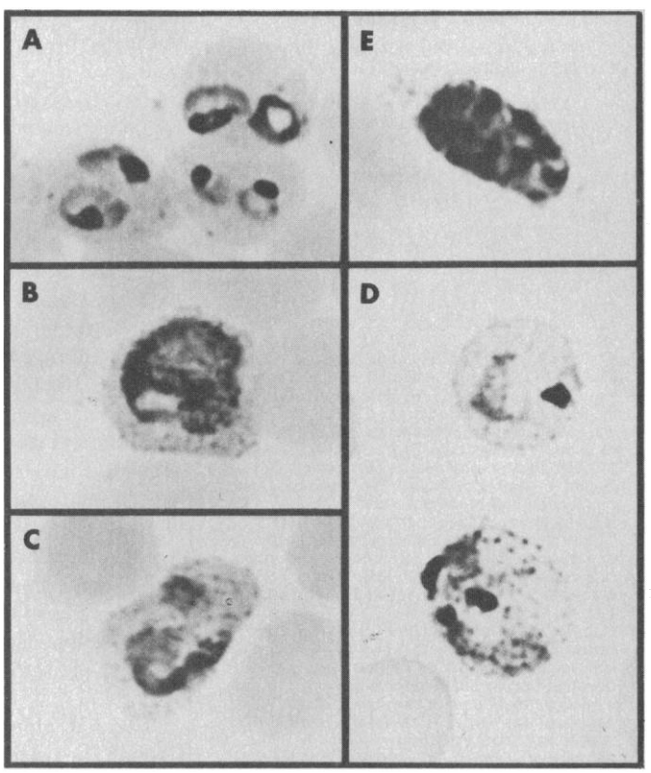

Fig. 1. *Plasmodium cynomolgi*. (A) Rings, (B and C) trophozoites, (E) schizont after 24 days in vitro, and (D) trophozoites in the peripheral blood of a rhesus monkey 11 days after inoculation with cultured *P. cynomolgi* (×2606).

only rarely encountered, had a normal morphology. Trophozoites in culture were less ameboid (Fig. 1, B and C) than those in vivo. This ameboid behavior appeared reversible, since the rhesus monkeys inoculated with cultured parasites developed infections where the trophozoites were characteristically highly ameboid (Fig. 1D); when their infected blood was used to reinitiate cultures, the trophozoites developing in vitro were again less ameboid.

Plasmodium cynomolgi differs from previously cultured malaria parasites in its potential for causing relapses. This parasite will be particularly suitable for combined studies in vivo and in vitro on relapsing malaria, since monkeys infected with *P. cynomolgi* show predictable relapses, unlike monkeys infected with *P. vivax* (*13*). Previously cultured malaria parasites include the falciparum-type *P. fragile* (*14*) and the malariae-type *P. inui* (*15*), both of which infect rhesus monkeys. The addition of *P. cynomolgi* underlines the value of *M. mulatta* as a simian model for studies on human malarias.

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8. Trade names and commercial sources are for identification purposes only and do not constitute endorsement by the Public Health Service or by the U.S. Department of Health and Human Services.
9. Prior to being used, human serum samples were tested by overnight incubation with rhesus monkey erythrocytes under routine culture conditions; the occasional samples causing erythrocyte agglutination were rejected. Growth of the parasites was better with human serum than with rhesus monkey serum.
10. Although *P. cynomolgi* can infect man [see (*3*)] attempts to maintain the parasite in vitro with human erythrocytes (O+, Duffy +) have thus far been unsuccessful.
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Long-Term Potentiation in the Hippocampal Slice: Evidence for Stimulated Secretion of Newly Synthesized Proteins

Abstract. *Long-term potentiation of the hippocampal slice preparation results in an increase in the incorporation of labeled valine into the proteins destined for secretion into the extracellular medium. Double-labeling methods established that the increased secretion of the labeled proteins was limited to the potentiated region of a slice; incorporation of labeled valine was increased in the hippocampus if potentiation was through the Schaffer collaterals and in the dentate if potentiation was through the perforant path. Controls for nonspecific stimulation showed no changes. There appears to be a link between long-term potentiation and the metabolic processes that lead to protein synthesis in the hippocampal slice system.*

Long-term potentiation (LTP) of synaptic responses in the rat hippocampus is used as a model system for the neurophysiological study of plasticity (*1–4*). Afferent stimulation of transverse sections of hippocampal slices of rat brain by application of a brief tetanizing train can produce a postsynaptic response that is two to ten times greater than the response before tetanus. The LTP lasts for weeks in the intact preparation (*1*) and for up to 10 hours in the in vitro preparation, its longevity being limited by the slicing technique (*2*). Biochemical effects in brain tissue after electrical stimulation have been demonstrated (*5*), and the effects of LTP on the pattern of protein phosphorylation have been studied (*6*).

We now report a relation between LTP and protein synthesis, explored by techniques that we used to study the pattern of protein synthesis in the goldfish brain (*7*) after training. In the goldfish, we found that two specific glial proteins are rapidly labeled and released into the extracellular fluid after the animals acquire a new pattern of behavior (*8*). Such changes are considered to be part of the sequence of metabolic events that ultimately lead to neuronal plasticity. If LTP in the hippocampal slice is also an indicator of neuronal plasticity, then by analogy to the goldfish data (*8*) some changes in the pattern of synthesis and secretion of proteins might be expected in the rat hippocampal slice after LTP.

Using a double-labeling procedure (*7*) in which the pattern of protein synthesis in a single potentiated slice of rat hippocampus is compared with that of an unpotentiated control slice, we have found a substantial increase in the secretion of labeled proteins into the extracellular fluid after LTP.

Transverse hippocampal slices, cut to preserve the organization of the hippocampus, were prepared from male Sprague-Dawley rats (250 g) (*9*). The slices were placed on nylon nets above a sample chamber containing 200 μl of Earle's incubation medium and maintained at 34°C in a humidified atmosphere of 95 percent O_2 and 5 percent CO_2. Such slices were capable of producing stable orthodromic responses for at least 8 hours. Stimuli were delivered with bipolar electrodes placed on the Schaffer collateral or the perforant path fibers. Monosynaptic excitatory postsynaptic potentials were recorded with electrodes at the CA1 region of the hippocampus or at the dentate gyrus. These electrode configurations record the monosynaptic population spike response to stimulation of afferent fibers.

Before being stimulated with tetanizing pulses, the slices were incubated for 1 hour and the input-output (I-O) function was determined (Fig. 1). The I-O function describes the relation between the magnitude of the afferent input and the amplitude of the response of the population of cells activated; it is thus a description of the synaptic efficacy or throughput of the system. The tetanus (33 Hz for 3 seconds) was produced at an intensity sufficient to evoke a population spike of 1 mV (*2*). An I-O curve determi-

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