Bulletin of the World Health Organization, 60 (4): 571 – 575 (1982)

Studies on serum requirements for the cultivation of *Plasmodium falciparum*. 2. Medium enrichment*

A. A. DIVO[1] & J. B. JENSEN[2]

Previous experiments using RPMI 1640 medium have indicated that the dialysis of human serum removes components of low relative molecular mass (6000 – 8000 RMM) that are essential for continuous cultivation of Plasmodium falciparum. *To determine which low-RMM components are important for parasite development, we compared growth in normal serum to that in dialysed serum using a number of other commercially available media, which we considered to be richer than RPMI 1640. Through these comparisons, we determined that hypoxanthine was the major dialysable nutrient required for parasite development. High quality bovine serum requires* $3 - 12 \times 10^{-5}$ *mol/litre of hypoxanthine as a supplement to support continuous cultures of* P. falciparum. *Thus far we have been unable to attain parasite growth in medium containing supplemented bovine serum that is as good as growth in medium containing human serum.*

The continuous cultivation of *Plasmodium falciparum* at present requires the addition of 50 ml of pooled human serum per litre of culture medium (5% serum) to ensure optimum parasite growth (*1*). This requirement for human serum reflects the inability of the basic culture medium, RPMI 1640, to meet the nutritional or regulatory needs of the parasite. By comparison with other cell culture techniques, the cultivation of *P. falciparum* is still in its infancy. Until the development of the Trager-Jensen method (*2*) for the continuous cultivation of the parasite, most research on basic parasite biology of *Plasmodium* spp. was carried out using animal models for study. Only a limited number of reports have addressed the nutritional status of *P. falciparum* (*3 – 8*).

Reports indicate that human serum may be replaced in media for continuous culture by supplemented bovine serum (*4 – 6*), but our experience is that parasite growth is inferior to that obtained using human serum. By more thoroughly defining the nutritional requirements of *P. falciparum*, it may be possible to eliminate the human serum now required for continuous cultivation, or to use animal sera without any reduction in growth. The advantages of using a system free of human serum have been discussed previously (*1*).

Since it has been shown that dialysed human serum is lacking in components with low relative molecular mass (RMM) that are necessary for parasite development (*3*), it is now possible to determine some of the factors required for parasite development by using dialysed serum supplemented with low RMM nutrients. We have also examined other commercially available culture media that are nutritionally richer than RPMI 1640, and have supplemented these media with dialysed human and selected animal sera as well as selected nutrients.

MATERIALS AND METHODS

Continuous cultures of *P. falciparum*, strain FCR3 (*9*), were maintained using the Petri dish–candle jar method (*10*). All experiments were begun using parasites grown in RPMI 1640[a] supplemented with 50 ml of type A Rh^+, pooled human serum per litre (5% serum) (*1*). To begin each experiment a common pool of 0.1% parasitized blood was divided into test groups of 4 dishes each. Experiments were monitored over a 96-h period, the culture medium being renewed once daily. Parasitaemias were determined by counting parasites per 10 000 erythrocytes.

Human and animal sera

The sera used included pooled human serum (PHS), and pooled lots of freshly collected bovine

* Michigan Agricultural Experiment Station journal article No. 10023. From the Department of Microbiology and Public Health, Michigan State University, East Lansing, MI 48824, USA. This investigation received the financial support of the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases.

[1] Graduate Assistant.

[2] Assistant Professor.

[a] Gibco Laboratories, Grand Island, NY, USA.

(PBS), porcine (PPS), and goat (PGS) sera. All sera were collected and pooled as reported by Divo & Jensen (*1*).

Media and supplements

The preparation of RPMI 1640 (hereafter referred to as RPMI) has been previously described by Jensen (*10*). Ham's F12 medium[b] (hereafter referred to as F12) with L-glutamine was prepared by dissolving 10.6 g of powdered medium in 900 ml of three times glass-distilled water; 5.94 g of HEPES and 1.0 g of glucose were added and dissolved; the total volume was brought to 1000 ml and 1.176 g of $NaHCO_3$ were added. Medium 199 with Earle's salts[b] and L-glutamine (hereafter referred to as 199E) was prepared by dissolving 9.9 g of medium in 900 ml of water; 5.94 g of HEPES and 1.0 g of glucose were added; the volume was brought to 1000 ml and 2.2 g of $NaHCO_3$ were added. Medium 199 with Hanks' salts[b] and L-glutamine (hereafter referred to as 199H) was prepared by dissolving 11.0 g of medium in 900 ml of water; 5.94 g of HEPES and 1.0 g of glucose were added; the volume brought to 1000 ml and 0.35 g of $NaHCO_3$ was added. The media were all adjusted to pH 7.2 – 7.4 using 10 mol/litre NaOH.

The following reagents were prepared in three times glass-distilled water at 100× the concentration required in the final medium. When preparing supplemented medium the volume of water initially added was reduced by the corresponding volume of the supplements to be added. The reagents and 100× concentrations were as follows: inosine (0.805 g/l), adenosine (0.802 g/l), adenine (0.405 g/l), hypoxanthine (0.41 g/l), L-proline (3.45 g/l), L-alanine (0.89 g/l), sodium pyruvate (11 g/l), vitamin B 12 (0.13 g/l), putrescine hydrochloride (0.0158 g/l), linoleic acid (0.0084 g/l—0.1 g dissolved in 1 ml of absolute ethanol, then diluted with water), alpha-lipoic acid (0.021 g/l—0.0106 g dissolved in 6 drops of 1 mol/litre NaOH, then diluted with water), $FeSO_4.7HOH$ (0.0834 g/l), $CuSO_4.5HOH$ (0.00025 g/l), and $ZnSO_4$ (0.086 g/l). A solution of 150 g of Neopeptone[c] per litre of water was prepared and used at a concentration of 12 ml per litre of RPMI 1640 (*4*). Media and reagents were sterilized by filtration.

Dialysis of human serum and Neopeptone

The dialysis of human serum was carried out using dialysis tubing (Spectrapor) with a 6000 – 8000 relative molecular mass pore size. Twenty ml of pooled human serum (PHS) were exhaustively dialysed for 48 h against two volumes of 2000 ml each of RPMI 1640 plus HEPES and $NaHCO_3$, the dialysing medium being renewed after 24 h. Twenty ml of Neopeptone (150 g/litre) were dialysed in the same manner. Control PHS and Neopeptone were minimally dialysed against 10 ml of the same medium in a 50-ml graduated cylinder. Dialysis was carried out at 4 °C.

[b] Gibco Laboratories, Grand Island, NY, USA.

[c] Difco Laboratories, Detroit, MI, USA.

RESULTS AND DISCUSSION

As indicated in Table 1, RPMI and F12 media were superior to the two 199 media tested when 10% pooled human serum (PHS) was used. In addition, exhaustively dialysed human serum was not effective when used to supplement RPMI, but was not so poor when used with F12 medium and the two 199 media. When human serum was dialysed against F12 medium and then used to supplement RPMI it remained effective. Since Jensen (*3*) has previously shown that dialysis of human serum results in the loss of factors of low relative molecular mass (less than 6000 – 8000 RMM) which are essential for parasite growth, these results indicate that F12 and the 199 media appear to contain factors that promote parasite growth which are not present in RPMI. Because F12 appeared to be the better medium under these conditions it was used to make further comparisons. The factors present in F12 but not in RPMI were used to supplement RPMI. These included $ZnSO_4$, $FeSO_4.7HOH$, alpha-lipoic acid, linoleic acid, putrescine hydrochloride, hypoxanthine, L-proline, L-ana-

Table 1. Growth of *P. falciparum* in different media, each supplemented with 10% pooled human serum, either minimally or exhaustively dialysed

Media	% Parasitaemia		% Reduction in growth between control and dialysed PHS
	Control PHS[a]	Exhaustively dialysed PHS[a]	
RPMI 1640	3.6 ± 0.2	1.2 ± 0.1	67
Ham's F12	4.4 ± 0.4[b]	3.7 ± 0.2	16
199 with Earle's Salts	2.1 ± 0.1	1.7 ± 0.1	19
199 with Hank's Salts	1.5 ± 0.1	1.3 ± 0.1	17

[a] Exhaustive dialysis was 1 : 10 000 whereas control sera were minimally dialysed 1 : 0.5 (Mean ± SD for 4 observations).

[b] This result indicates that F12 is superior to RPMI when using minimally dialysed PHS, but from our experience we cannot conclude that F12 is superior to RPMI when using normal non-dialysed PHS.

line, and sodium pyruvate. Of all the supplements tested (individually and in various combinations), only hypoxanthine was found to contribute to increased parasite growth.

The data in Table 2 indicate that the addition of hypoxanthine to RPMI restored parasite growth in exhaustively dialysed PHS to the level seen before dialysis. The results infer that hypoxanthine was the primary component required for parasite growth that was removed by exhaustive dialysis, and that the serum concentration of purines is critical for parasite development. These conclusions are supported by Webster et al. (*11*) who demonstrated quantitatively that the concentration of purines, primarily hypoxanthine, in serum-supplemented medium decreases significantly during parasite growth.

Table 2. Comparison of *P. falciparum* growth in RPMI 1640, with PHS or exhaustively dialysed PHS with or without hypoxanthine

Media	% Parasitaemia[a]	% Reduction in growth following dialysis
RPMI 1640 + control PHS	3.8 ± 0.2	–
RPMI 1640 + exhaustively dialysed PHS		
without hypoxanthine	0.8 ± 0.1	78
with hypoxanthine[b]	3.5 ± 0.2	8

[a] Mean ± SD for 4 observations.
[b] The concentration of hypoxanthine was 3×10^{-5} mol/l.

To determine whether F12 offered any advantages for parasite cultivation when other animal sera are used, 5% PHS was compared with 10% PBS (pooled bovine serum), 10% PGS (pooled goat serum), 10% PPS (pooled porcine serum), and a combination of all three. The data in Table 3 indicate that F12 supplemented with 10% PBS was superior to the other sera tested, and that only PBS would support continuous parasite growth. These results were interesting in view of our previous findings using RPMI supplemented with 10% PBS and Neopeptone (*1*), which demonstrated that, of a number of animal sera tested, only bovine serum supplemented with Neopeptone would support continuous parasite growth. In this instance, F12 plus PBS did not require any Neopeptone to support the falciparum cultures.

As indicated by the data in Table 4, RPMI plus 5% PHS was generally superior to F12 supplemented with 5% PHS; this confirms all our previous experience. In addition, RPMI is cheaper and easier to prepare.

Table 3. Comparison of *P. falciparum* growth in Ham's F12 medium with different serum supplements

Supplement added to Ham's F12 medium	% of growth obtained in 5% PHS[a]	Comments
5% PHS	100 ± 4.6	Represents a 40× increase in parasitaemia over 96 h
10% PBS	60.9 ± 4.6	Supported continuous parasite growth[b]
10% PPS	27.6 ± 2.8	Subsequent subcultures failed
10% PGS	NAG[c]	
10% PBS/PPS/PGS	53.9 ± 2.1	Subsequent subcultures failed

[a] Mean ± SD for 4 observations.
[b] Experiment terminated after 100 days.
[c] No appreciable growth.

Table 4. Comparison of *P. falciparum* growth in RPMI 1640 and Ham's F12 media, with different supplements

Medium	% of growth obtained in RPMI 1640 + 5% PHS[a]
RPMI + 5% PHS	100 ± 2.8
F12 + 5% PHS	87.6 ± 5.8
RPMI + 10% PBS	6.2 ± 1.0
F12 + 10% PBS	55.1 ± 3.7
RPMI + 10% PBS + Neopeptone[b]	68.4 ± 3.4
F12 + 10% PBS + Neopeptone[b]	46.8 ± 2.6

[a] Mean ± SD for 4 observations.
[b] 12 ml/litre of medium, of a solution of 150 ml of Neopeptone/litre of water.

Although F12 was not superior to RPMI when using PHS, when both media were supplemented with 10% PBS, F12 was by far superior to RPMI but still inferior to RPMI + PHS. When Neopeptone was used with the PBS, it contributed significantly to parasite development in RPMI, but appeared to detract from parasite growth when used in F12. The results suggest that a factor required for parasite growth may be present in both Neopeptone and Ham's F12.

To determine the factors present in F12 that accounted for its ability to support continuous parasite growth when supplemented with PBS, RPMI was supplemented with the components that are present in F12 but not in RPMI. The components tested are

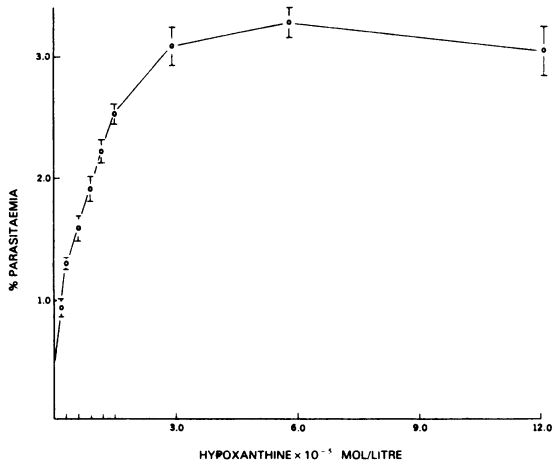

Fig. 1. Parasitaemia obtained with *P. falciparum*, strain FCR3, in RPMI 1640 + 10% PBS with the addition of different concentrations of hypoxanthine (Mean ± SD for 4 observations).

Table 5. Growth of *P. falciparum* in 10% PBS, using RPMI supplemented with minimally or exhaustively dialysed Neopeptone, and the effect of supplementation of the medium with hypoxanthine[a]

Medium	% of growth obtained in 5% PHS[a,b]
RPMI + 5% PHS	100 ± 4.6
RPMI + 10% PBS + control Neopeptone	60.6 ± 3.2
RPMI + 10% PBS + exhaustively dialysed Neopeptone	
without hypoxanthine	10.2 ± 1.0
with hypoxanthine	80.9 ± 2.9
RPMI + 10% PBS + hypoxanthine	78.8 ± 5.9

[a] Hypoxanthine was used at a concentration of 6×10^{-5} mol/l.
[b] Mean ± SD for 4 observations.

listed on pages 572 – 573, and again the only supplement that promoted parasite growth in RPMI + PBS was hypoxanthine. To determine the optimum concentration of hypoxanthine, a titration curve was constructed (Fig. 1). The data indicate that the optimum concentration lies between 3×10^{-5} mol/litre and 12×10^{-5} mol/litre—the upper limit was not determined. Twice the concentration found in F12 (6×10^{-5} mol/litre) was used for making further comparisons. The results indicated that PBS was deficient in utilizable purines, and that these must be added in order to obtain continuous parasite growth. To determine whether Neopeptone was acting as a purine source for parasite growth, it was dialysed in the same manner as described above for PHS.

The data in Table 5 indicate that exhaustively dialysed Neopeptone was not effective when used to supplement PBS, and that hypoxanthine restored its ability to support parasite growth. Supplementation with hypoxanthine resulted in parasite growth that was superior to that obtained with minimally dialysed Neopeptone. When normal, undialysed, Neopeptone was compared with hypoxanthine as a PBS supplement, hypoxanthine always supported better parasite growth. Our previous work (*1*), using high quality bovine serum supplemented with Neopeptone, was reproducible using hypoxanthine instead of Neopeptone.

We have found that inosine, adenosine, and hypoxanthine will supplement PBS equally well, and that adenine will support parasite growth to a lesser degree. These results are supported by Webster et al. (*11*) who have described the quantitative relationships between purines, *P. falciparum*, and the erythrocyte. They reported the concentration of hypoxanthine, in complete RPMI 1640 (RPMI + 10% human serum) to be $1.5 - 3.0 \times 10^{-5}$ mol/litre, with the concentrations of the other purines being much lower (less than 2×10^{-6} mol/litre). It has been reported that the concentration of hypoxanthine in bovine plasma is 6×10^{-7} mol/litre and that of adenosine 5×10^{-8} mol/litre (*12*). When formulated into complete medium with 10% PBS, the approximate purine concentration would be 6.5×10^{-8} mol/litre, or between 250 – 500× less than when 10% human serum is used, assuming that the plasma concentration of hypoxanthine reflects the PBS concentration. The concentration of hypoxanthine that we found to be optimum covered the range $1.5 - 12.0 \times 10^{-5}$ mol/litre, which would include the values reported by Webster et al. (*11*) for the concentration of purines in RPMI with 10% human serum added.

Ifediba & Vanderberg (*4*), Zhengren et al. (*5*), and Siddiqui (*6*) have all reported methods for the continuous cultivation of *P. falciparum* in bovine serum, and their success may be explained by the fact that the media they described all contained purines or Neopeptone. Ifediba & Vanderberg's work with Neopeptone has been discussed. Zhengren et al. reported using calf serum with medium 199, which contains significant concentrations of hypoxanthine, adenine, and guanine. Siddiqui reported that RBC extract and bovine serum in RPMI would support parasite growth and it is commonly known that the erythrocyte extract contains a high concentration of purines.

In summary we have determined that hypoxanthine is the major dialysable component in human serum that is essential for the continuous cultivation of

P. falciparum, and that freshly collected, pooled adult bovine serum (PBS) when supplemented with hypoxanthine will result in better growth when compared with PBS supplemented with Neopeptone. We also found that there was no advantage in using F12 or either of the 199 media when human serum was used as a supplement. So far we have not been able to reduce the pooled human serum below 5% by addition of hypoxanthine, indicating that at this serum concentration some other growth factor becomes limiting. In our experience, PBS supplemented with hypoxanthine supports continuous parasite growth at 60–70% of the growth rate in 5% PHS, and even after 4 months of continuous cultivation in PBS, parasite growth was still not equal to that usually seen with human serum.

RÉSUMÉ

ÉTUDES SUR LES BESOINS EN SÉRUMS POUR LA CULTURE DE *PLASMODIUM FALCIPARUM*. 2. MILIEUX ENRICHIS

Des expériences antérieures ont montré que la dialyse du sérum humain élimine les constituants à faible masse moléculaire relative (6000–8000) qui sont essentiels à la culture continue de *P. falciparum*; dans ces expériences, le sérum dialysé a été ajouté au milieu de culture RPMI 1640. Pour déterminer quels sont les constituants à faible masse moléculaire relative importants pour le développement du parasite, nous avons également comparé sa croissance en ajoutant du sérum normal et du sérum dialysé à divers autres milieux de culture disponibles dans le commerce, que nous estimons plus riches que le RPMI 1640. Nous avons constaté que le RPMI 1640 était supérieur aux autres milieux soumis aux épreuves lorsqu'on utilisait du sérum normal, mais que le milieu Ham F12 et le milieu 199 additionné de sels Hanks et Earle lui étaient supérieurs lorsqu'on utilisait du sérum dialysé. En ajoutant au RPMI 1640 certains constituants présents dans les autres milieux mais non présents dans le RPMI 1640, nous avons déterminé que l'hypoxanthine était le principal nutriment dialysable qui est nécessaire pour la croissance du parasite.

Nous avons également comparé la croissance du parasite dans le milieu RPMI 1640 avec sa croissance dans le milieu Ham F12 lorsqu'ils sont additionnés de sérums de bovins adultes, de porcins et de caprins fraîchement recueillis et stockés. Le milieu Ham F12 s'est montré capable de favoriser la croissance continue du parasite lorsqu'il est additionné de sérum bovin, mais non lorsqu'on y ajoute du sérum porcin ou caprin. De plus, en ajoutant au RPMI 1640 des constituants présents dans le Ham F12 mais non présents dans le RPMI 1640, nous avons déterminé que le sérum bovin additionné d'hypoxanthine ($3-12\times10^{-5}$ mol/litre) favorisait la croissance continue du parasite. D'autres rapports décrivent la culture continue avec un supplément de sérum bovin plus divers autres nutriments; nos résultats indiquent que ces nutriments servent probablement de source de purine, laquelle est indispensable pour le développement du parasite. L'hypoxanthine ajoutée au supplément de sérum bovin permet d'obtenir des cultures continues du parasite, mais à un niveau réduit en comparaison avec la croissance qui se produit lorsqu'on utilise du sérum humain dans le milieu de culture. L'adjonction d'hypoxanthine au sérum humain n'améliore pas la croissance du parasite et ne permet pas non plus l'utilisation de concentrations inférieures de sérum.

REFERENCES

1. DIVO, A. A. & JENSEN, J. B. *Bulletin of the World Health Organization*, **60**: 565–569 (1982).
2. TRAGER, W. & JENSEN, J. B. *Science*, **193**: 673–675 (1976).
3. JENSEN, J. B. *Bulletin of the World Health Organization*, **57** (Suppl. 1): 27–31 (1979).
4. IFEDIBA, T. & VANDERBERG, J. P. *Journal of parasitology*, **66**: 236–239 (1980).
5. ZHENGREN, C. ET AL. *Chinese medical journal*, **93**: 31–35 (1980).
6. SIDDIQUI, W. A. Continuous *in vitro* cultivation of *Plasmodium falciparum* in human erythrocytes: Description of a simple technique to obtain high yields of parasites. In: Maramorosch, K. & Hirumi, H., ed., *Practical tissue culture applications*, New York, Academic Press, 1979, pp. 267–277.
7. DRUILHE, P. ET AL. *Tropenmedizin und Parasitologie*, **31**: 409–413 (1980).
8. BUTCHER, G. A. *Bulletin of the World Health Organization*, **57** (Suppl. 1): 17–26 (1979).
9. JENSEN, J. B. & TRAGER, W. *American journal of tropical medicine and hygiene*, **27**: 743–748 (1978).
10. JENSEN, J. B. & TRAGER, W. Journal of parasitology, **63**: 883–886 (1977).
11. WEBSTER, H. K. ET AL. *Clinical research*, **28**: 326–336 (1980).
12. SEIDER, M. J. & KIM, H. D. *American journal of physiology*, **235**: C262–C267 (abstr.) (1979).