IMPROVED IN VITRO CULTURE OF PARASITIC FRESHWATER MUSSEL GLOCHIDIA

A report of research conducted for

The Tennessee Valley Authority

for the purpose of

improving current freshwater mussel glochidial culture used for transforming glochidia to juveniles

by

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December 4, 1990

INTRODUCTION

Freshwater mussel glochidia (larvae) are naturally transformed into juveniles, which will grow into adults, via encystment in fish tissue. This results in the development of their internal organs necessary for self-sustained existence as benthic organisms. Interest in enhancing production of mussels through the use of artificial culture was seen as early as the 1920's when Ellis and Ellis (1926) reported the successful culture of glochidia to transformation when these were excised from the gill tissue of their fish host. The details of their solutions were not published, and the removal from the host fish may have actually stimulated this development process prior to culture initiation.

Finally, in the early 1980's, interest in the artificial culture of glochidia was revived by Isom and Hudson (1982) when they reported success in the transformation of several species without the use of a fish host at any time in the process. This technique, which began as a modification of modern cell-culture techniques, made use of a mixture of amino acids, vitamins, and glucose in a unionid Ringers solution along with the addition of fish plasma as a source of protein, growth stimulants, hormones, etc. Although this work began by mixing these components from scratch (using the concentrations found in fish plasma as a guideline for each component), Isom and Hudson (1982) also reported that pre-mixed, commercially available cell culture media (Eagle's MEM with essential and non-essential amino acids, and Medium 199) had nearly all of these amino acids in concentrations as high or higher than those found in fish plasma and worked well in culture.

Even though this mixture has been used to produce thousands of juvenile mussels for use in toxicology research (Wade et al., 1989), it has been less than convenient for other laboratories to use because of the requirement that the researcher has available a ready supply of fish and be able to drain their blood and subsequently separate its plasma. Because of this inconvenience and because the use of fish plasma introduces more variation in the results, our current research was initiated in an effort to develop; 1) a serum/plasma-free medium, or 2) a medium using commercially available serum in minimal concentrations.

Following the completion of our experimentation, a paper was published by Keller and Zam (1990) which partially addresses these problems. Although a representative of the consulting firm which held the grant to fund part of this research visited the T.V.A. aquatic toxicology lab at Brown's Ferry Nuclear Power Plant in Alabama and observed our use of standard, commercially available medium, one major thrust of their paper concerns the new use of commercially available medium. This represents no advancement over our standard practice for transforming glochidia (Isom and Hudson, 1982; Wade et al., 1989); furthermore, Isom and Hudson (1982) clearly state that they tried pre-mixed Eagle's MEM and Medium 199 with success. Keller and Zam did demonstrate that other sera could be substituted for the fish plasma with success.

MATERIALS AND METHODS

All comparisons were made using the modified standard medium described by Isom and Hudson (1982, 1984) as a control. This medium is comprised of an artificial portion containing Eagle's essential and non-essential amino acids (Eagle's MEM) in unionid Ringers with $NaHCO_3$ for pH control, vitamins, antibiotics and glucose. Fish plasma was added as the natural protein source in a final ratio of 2/3 MEM to 1/3 plasma (Isom and Hudson, 1982). All treatments and their control used glochidia from Anodonta imbecillis, a widespread species used in juvenile toxicity tests (Wade et al., 1989). These glochidia came from the Haleyville reservoir, Haleyville, Alabama, and ponds in Lawrence County, Alabama, and Cabarras County, North Carolina. Another species, never before cultured, Elliptio lanceolata, was collected from Duncan Creek, Laurens County, South Carolina, and cultured. Glochidia were removed as described by Isom and Hudson (1982); however, glochidia were rinsed in autoclaved river water rather than deionized water. Approximately 400 - 900 glochidia were seeded in 3 ml total medium in cell-culture dishes which were 60mm in diameter. Each treatment and control had three repetitions of these dishes, all of which were incubated at 23-24°C in an incubator having a 4.6% CO_2 atmosphere. Counts were made of developing and non-developing glochidia on day 1 and day 6 of the glochidial culture, then these were placed in freshwater culture on day 7 and counted again on day 8 (one day after placement in freshwater culture). Counts from days 6 and 8 were used for analysis.

Although day 6 counts represented the untouched glochidial culture which had no rinsing and could not have any error caused by the accidental removal of dead glochidial shells, they did have potential error in that, due to lack of movement, viability of individual glochidia was more difficult to discern. Day 8 allowed the counting of actively moving, transformed juveniles, but may have been inflated by the loss of glochidial shells when these were rinsed and transferred to water on day 7. By counting both days 6 and 8, a more valid picture is probably formed. These counts were made by two observers, one using a Zeiss inverted compound microscope at 100X magnification and the other using a Baush and Lomb dissecting microscope at 30X magnification. A total of 100 glochidia were counted per dish by each observer, totaling 300 per observer per treatment per day. Since counts from day 6 and day 8 were averaged, a total of 1200 juvenile counts were made per treatment. (It is worthy of note that each count was a free sample from the dish population; therefore, an individual may have been counted more than once, either by different observers or on different days.)

The experimental design was somewhat truncated, since our early results determined the scope and direction of our later work. Experimentation began with a comparison of six sera versus fish plasma (two cultures), followed by combinations of fish with the best three of these sera, then the testing of serum replacements, alone and in combination with fish plasma, and finally in combination with the serum having the highest yield. Upon determination of the best combination, the components of this mixture were tested at different concentrations

to determine the best percentages for each. In all, fifteen cultures were initiated and over 60 treatments of different medium combinations were compared. The identities of these combinations are provided with their results in the next section. Fish controls were used for comparison in each culture.

These data were collected, as described previously, and the total number of developing glochidia in day 6 and day 8 was visually compared (figures) between treatments and control by converting the percent of developing glochidia in the control group of each culture (those in fish plasma) to a value of 100%. Then, the number of developing glochidia in day 6 and 8 in the treatment groups was converted to a value which represented their fraction of this control. In this way, all cultures would have a control with a standard value of 100%, creating a standardized value to allow each treatment group to be compared with those from other cultures. (This is necessary since transformation yields in culture controls vary from one to another, apparently due to the condition of the glochidia in the parent).

Global tests of the average actual counts (not standardized values) of all treatments in a given comparison group (i.e. comparing three sera and a control) were tested using contingency chi-square analysis. If this proved to be significantly different between the treatments and control when comparing the number of transformed vs. non-transformed juveniles, the control was compared with different individual treatments from the group to identify those with significant differences.

RESULTS AND DISCUSSION

COMPARISON OF SERA:

Six sera were initially compared with fish plasma in the first two cultures, and the top four again in culture III (figure 1). Fish plasma performed best in all three cultures with the exception of culture I, which had an extremely low yield and probably was not as suitable for comparison. Contingency chi-square analysis of the frequencies in culture II indicated that there was a significant difference between test groups (chi-square = 98.88 with 6 d.f.). Further comparison of fish (which performed the best) with the next best yield (rabbit serum) indicated a significant difference between these two (chi-square = 11.47 with 1 d.f.), demonstrating that fish transformation was significantly higher than all individual test groups. Culture III, which tested fish plasma against the top performing four sera from cultures I and II, indicated a significant difference when all sera and fish plasma were compared (chi-square = 58.17 with 4 d.f.). Further reduction showed significant differences until only fish plasma and rabbit serum remained in the comparison group, which were not significant (chi-square 2.14 with 1 d.f.). Our results differ from those of Keller and Zam (1990) in that horse serum performed below rabbit, porcine and fetal bovine sera in all cultures, and definitely below fish plasma (horse transformation yield ranged from 8.5 to 35 % of fish transformation yield). This seems to indicate that there must be

another factor, or other factors, involved in these comparisons. Keller and Zam (1990), despite their data showing horse and neonatal calf serum as being superior to fish plasma in table 1 of their paper (Neonatal calf = 95.5, Horse = 94.7 and fish = 81.8% transformation) also show in their table 2 that fish plasma performed the best (fish = 86.8, horse = 76 and neonatal calf = 60%, with the neonatal calf being significantly lower than the fish) under conditions identical to the cultures in the first table ($NaHCO_3$ buffer, 5% CO_2, 23°C). They made no comment concerning this discrepancy.

SERUM/PLASMA COMBINATIONS:

The best four sera were compared in combination with fish plasma (1:1 proportion) in culture III, and the the best two sera again in culture IV (figure 2). Note that combinations of fish with rabbit serum produced rates of transformation which were equal or better than the fish plasma control, while fish/porcine, fish/horse and fish/fetal bovine combinations were slightly lower than fish plasma. Comparison of yields from fish/rabbit, fish/porcine and rabbit/porcine combinations to the fish control in culture IV were not significantly different (chi-square = 5.81 with 3 d.f.); however, culture III results indicated fish/rabbit combinations were significantly higher than the fish control (chi-square = 4.1 with 1 d.f.) while those groups with fish/fetal bovine and fish/horse were significantly lower (chi-square = 6.99 and 4.83

Figure 1. Comparison of fish plasma with commercially available sera in cultures I, II, and III. (Fish controls = 100% transformation, all others relative.)

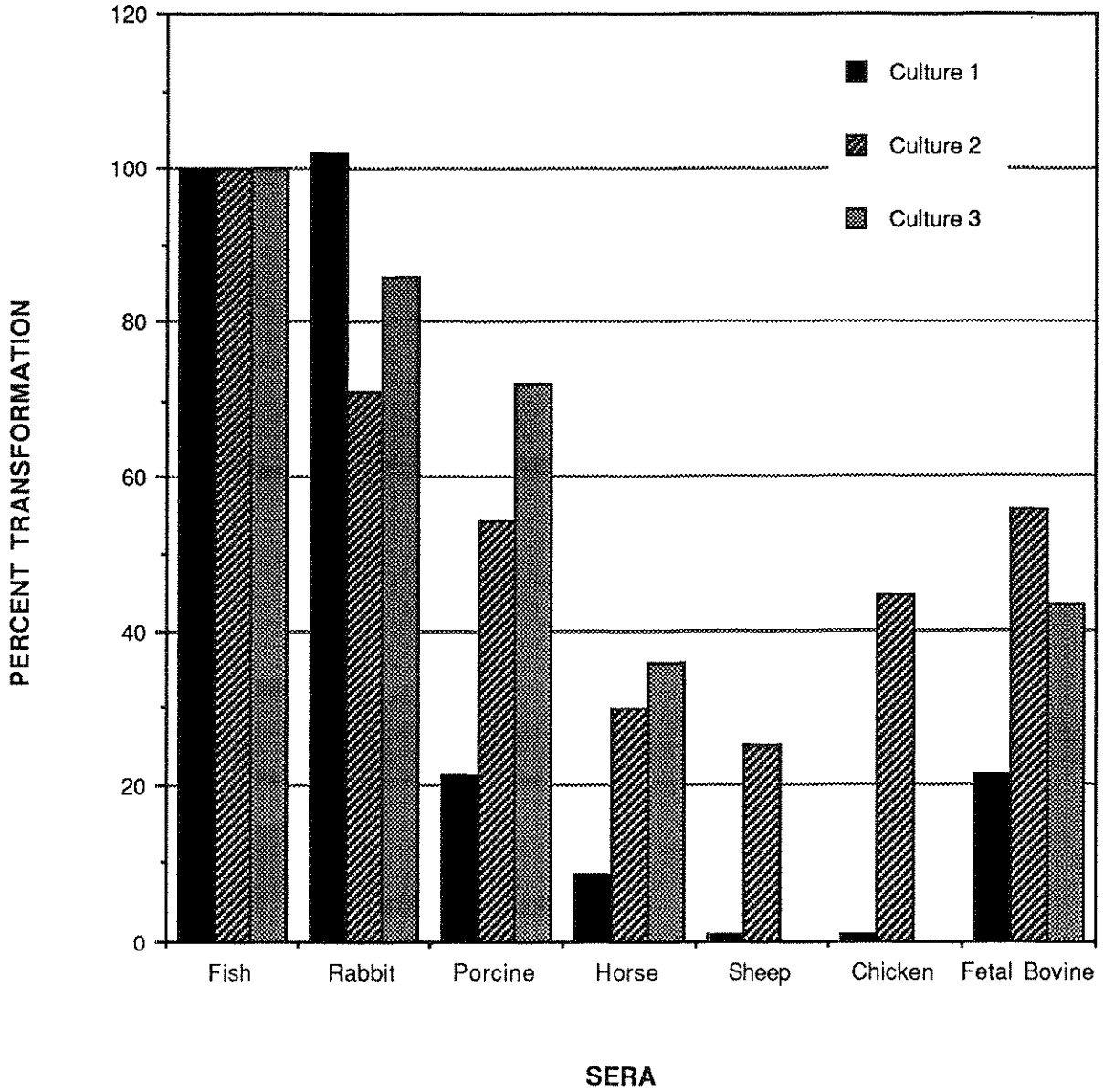
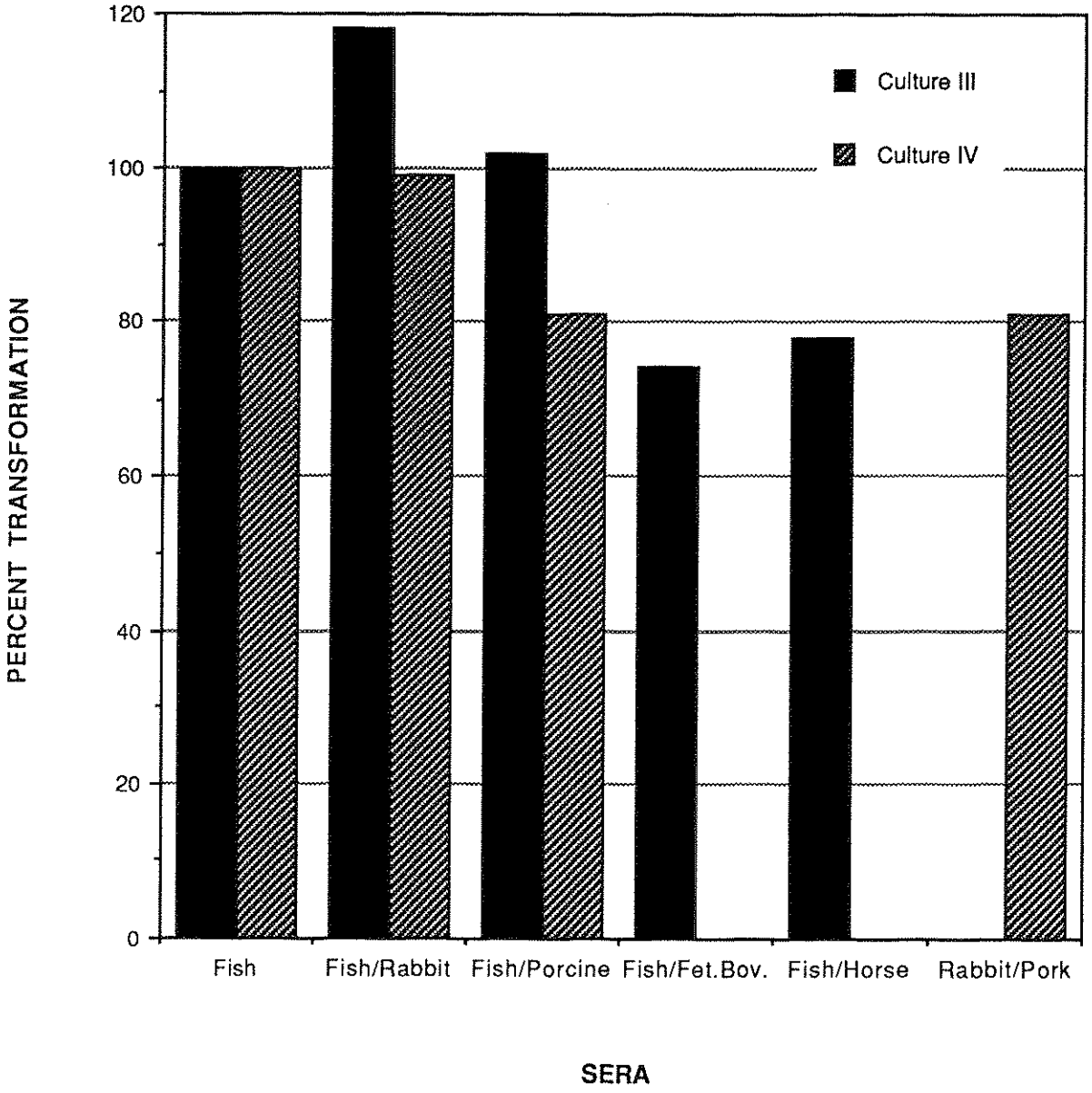


Figure 2. Comparison of combinations of fish plasma with the best transforming sera in cultures III and IV. (Fish controls = 100% transformation, all others relative.)



respectively with 1 d.f. each). As already stated, an attempt to compare the best two sera (rabbit and porcine) in combination without fish plasma also proved to be equal to the fish control.

SERUM-REPLACEMENTS:

Rabbit serum performed better than other commercially available sera in the above cultures; however, our goal was to reduce the use of plasma or serum as much as possible in glochidial culture while maintaining a high yield of transformation. Six serum replacements were tested, including one controlled process serum replacement (CPSR-5, Sigma), one bovine serum supplemented with growth enhancing components (Seru-Max, Sigma) and four low protein serum replacements (LPSR-1, Sigma; TCM, TM-235 and TCH supplements, Celox). These six were compared alone and in combination with fish plasma (1:1) to determine their transforming abilities.

When used alone, all of these serum replacements give poor yields (figure 3) which were significantly lower than the fish control (fish vs. CPSR, LPSR and Seru-Max in culture V had a chi-square = 167.41 with 3 d.f.; fish vs. TCH, TCM and TM-235 in culture VI had a chi-square = 145.12 with 3 d.f.); however, figure 4 shows that when used in combination with fish plasma (diluted as recommended and used 1:1), all produced results equal to the fish plasma (fish vs. fish/CPSR and fish/LPSR, chi-square = 1.55 with 2 d.f.; fish vs. fish/TCH, fish/TCM

Figure 3. Comparison of serum replacements for transforming abilities in cultures V and VI. (Fish controls = 100% transformation, all others relative.)

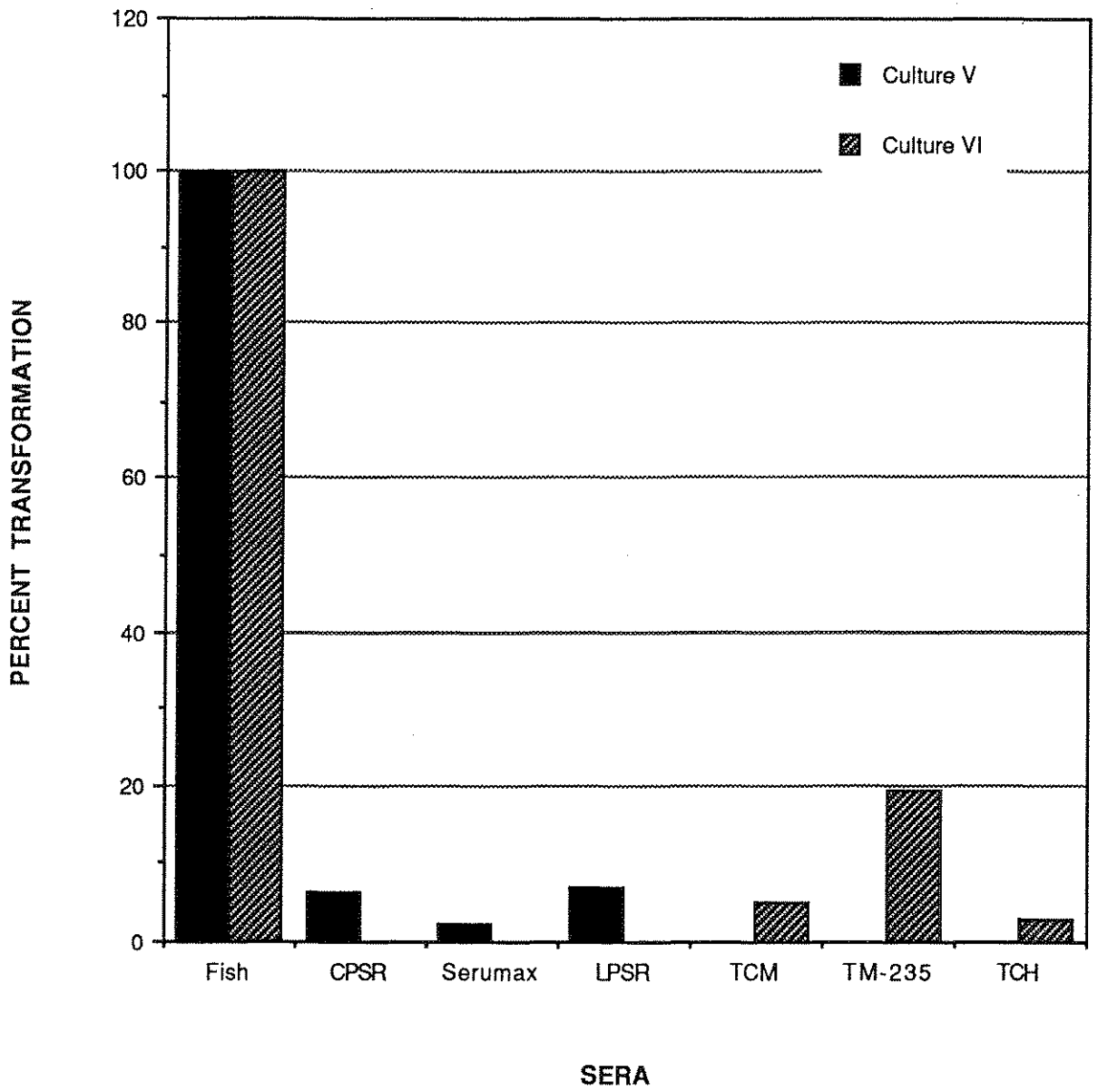
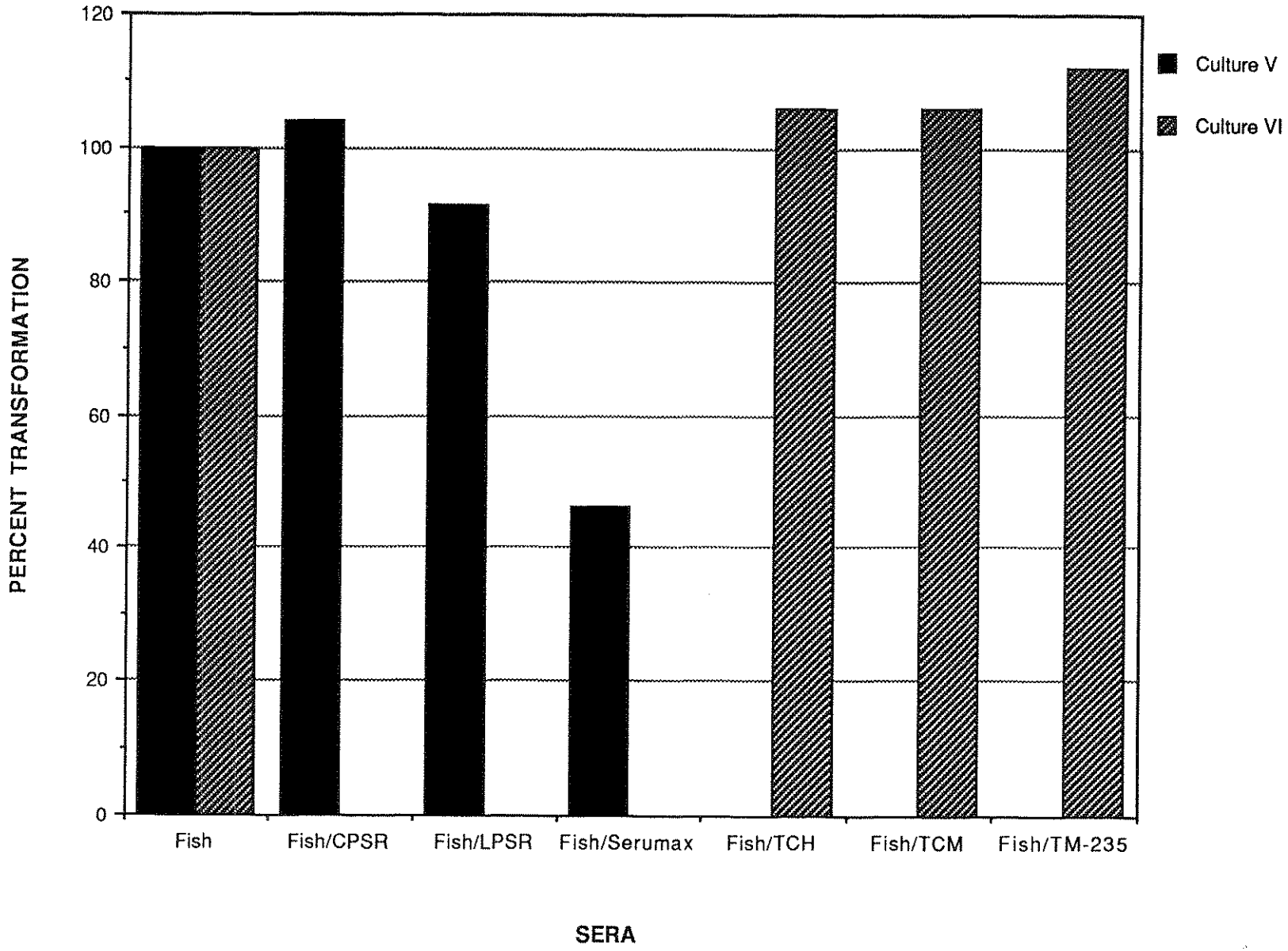


Figure 4. Comparison of serum replacements in combination with fish plasma in cultures V and VI. (Fish control = 100% transformation, all others relative.)



and fish/TM-235, chi-square = 1.13 with 3 d.f.), except the fish/Seru Max combination, which was significantly lower (chi-square of 24.84, 1 d.f.).

Since our goal was to replace the fish plasma, we now tested our best serum (rabbit) in combination with our best five serum replacements (TCH, TCM, TM235, CPSR AND LPSR) along with two combinations of serum replacements (TCH/CPSR, TM235/TCH) without any serum. None of these combinations worked very well (figure 5), but rabbit/TCH, followed by rabbit/TCM performed much better than any of the others (still significantly lower from fish control; chi-square = 11.08, 2 d.f.). As illustrated in figure 5, the serum replacements without serum transformed far lower than the the serum replacements with serum.

Since combinations of rabbit serum with any single serum replacement produced lower than desirable yields, we prepared more complex combinations using the top three performing sera (rabbit, porcine, fetal bovine) in combination with each other and the six serum replacements. As illustrated in figure 6, the highest yielding treatment was rabbit/TCH,TCM,TM235, followed closely by rabbit/CPSR,LPSR and rabbit, porcine/TCH,TCM,TM235 and rabbit, porcine/ CPSR, LPSR (all tested not significantly different from fish control with a chi-square = 5.66, 4 d.f.).

Since rabbit combined with TCH/TCM/TM235 and CPSR/LPSR performed the highest, another culture comparing rabbit serum in combinations with TCM/TCH, TCH/CPSR, TCH/LPSR and TCH/LPSR/CPSR was initiated. The rabbit/TCM,TCH and the rabbit/TCH,CPSR performed almost as well as the

Figure 5. Comparison of serum replacements in combination with rabbit serum, and each other, in culture VIII. (Fish control = 100% transformation, all others relative.)

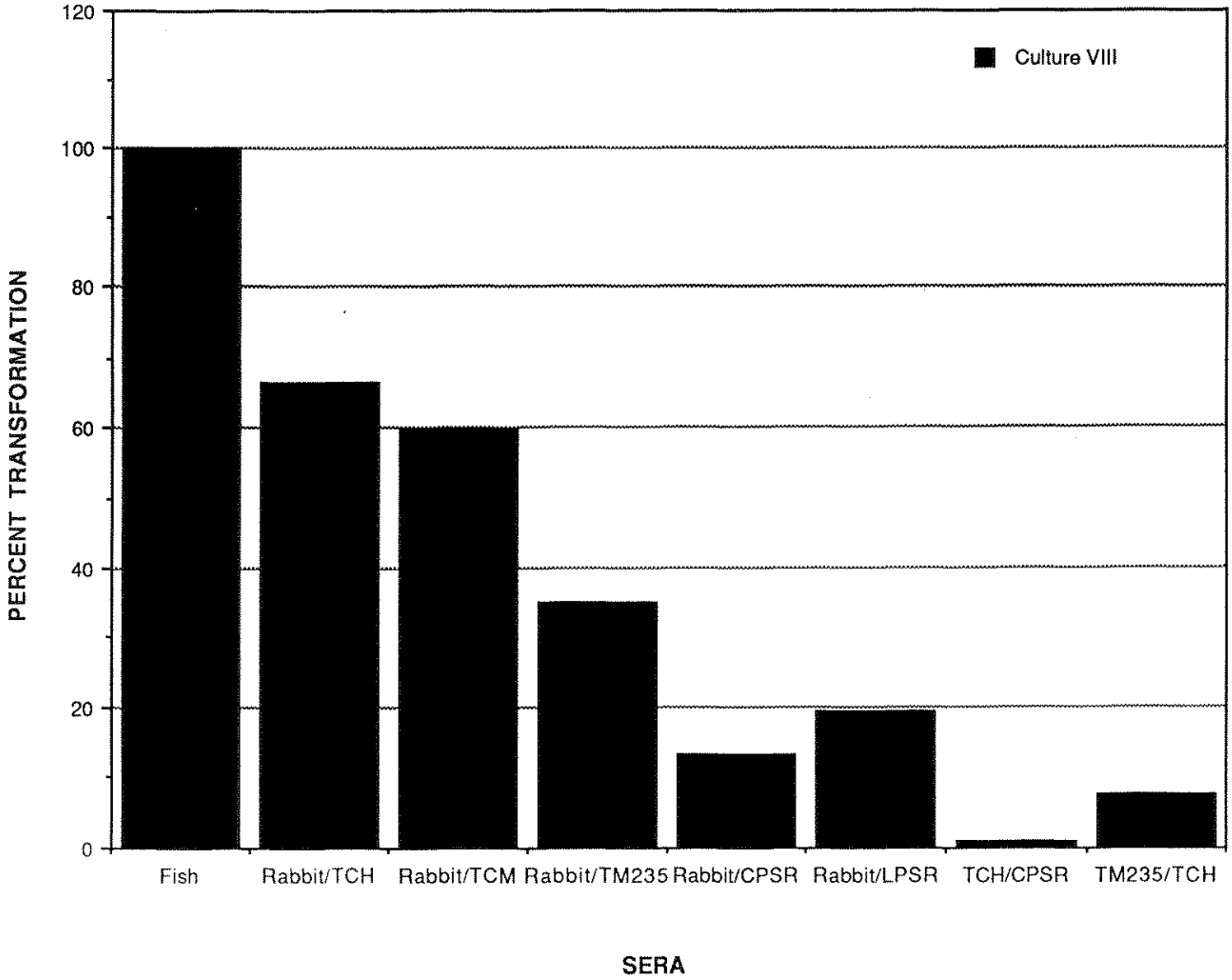
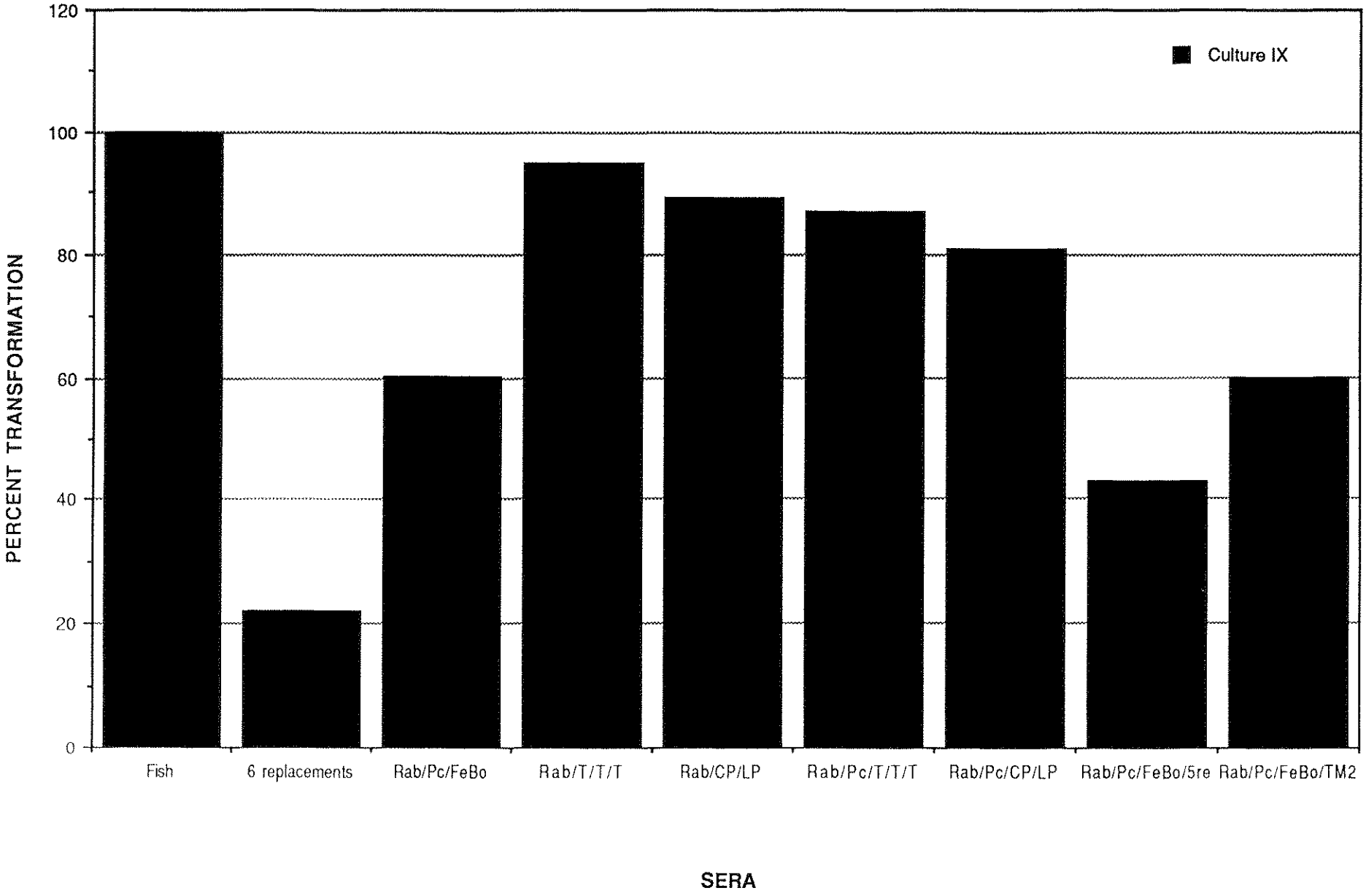


Figure 6. Comparison of serum replacements in combination with rabbit, porcine and fetal bovine sera, and each other, in culture IX. (Fish control = 100% transformation, all others relative.)



fish plasma (95 and 93% respectively), while the rabbit/TCH,LPSR and rabbit/TCH,LPSR,CPSR were 84% and 85% of fish transformation, respectively (figure 7). None of these combinations tested significantly different from the fish control.

Since the rabbit combinations with TCM/TCH were closest to the performance of the fish plasma, we designed a test to compare these two serum replacements in varying concentrations in mixtures with rabbit serum. The first culture, using stock TCM/TCH in equal proportions to each other, compared these in the following concentrations with rabbit serum: 100% rabbit serum; 67% rabbit/33% TCM/TCH; 50% rabbit/50% TCM/TCH; 33% rabbit/67% TCM/TCH; 100% TCM/TCH. The varying concentrations did not seem to matter (chi-square on fish control and first four tests = 2.22, 4 d.f.) until all of the rabbit serum was removed (significantly different from fish, chi-square = 29.94, 1 d.f.). Note that the 33% rabbit/67% TCM/TCH (actually a 1:1:1 ratio of rabbit, TCM and TCH respectively), although not significantly different, did perform slightly higher than the fish plasma control (figure 8).

A final comparison was made using these same proportions of rabbit and the two serum replacements (1:1:1 ratio), but varying the dilutions of the original stock of TCM and TCH. The original media were mixed using either TCM and/or TCH from a 12% concentration stock solution (0.6 ml of the TCM or TCH with 4.4 ml MEM in each stock). This was added to each serum in the experiment at a 1 serum:1 TCM:1 TCH ratio. Increasing stock solution concentrations of 24%, 48% and 100% each were mixed and added to rabbit serum at the above 1:1:1 proportions. For comparison, the 12% and 48% concentrations of TCM and

Figure 7. Comparison of serum replacements in combination with rabbit serum in culture X. (Fish control = 100% transformation, all others relative.)

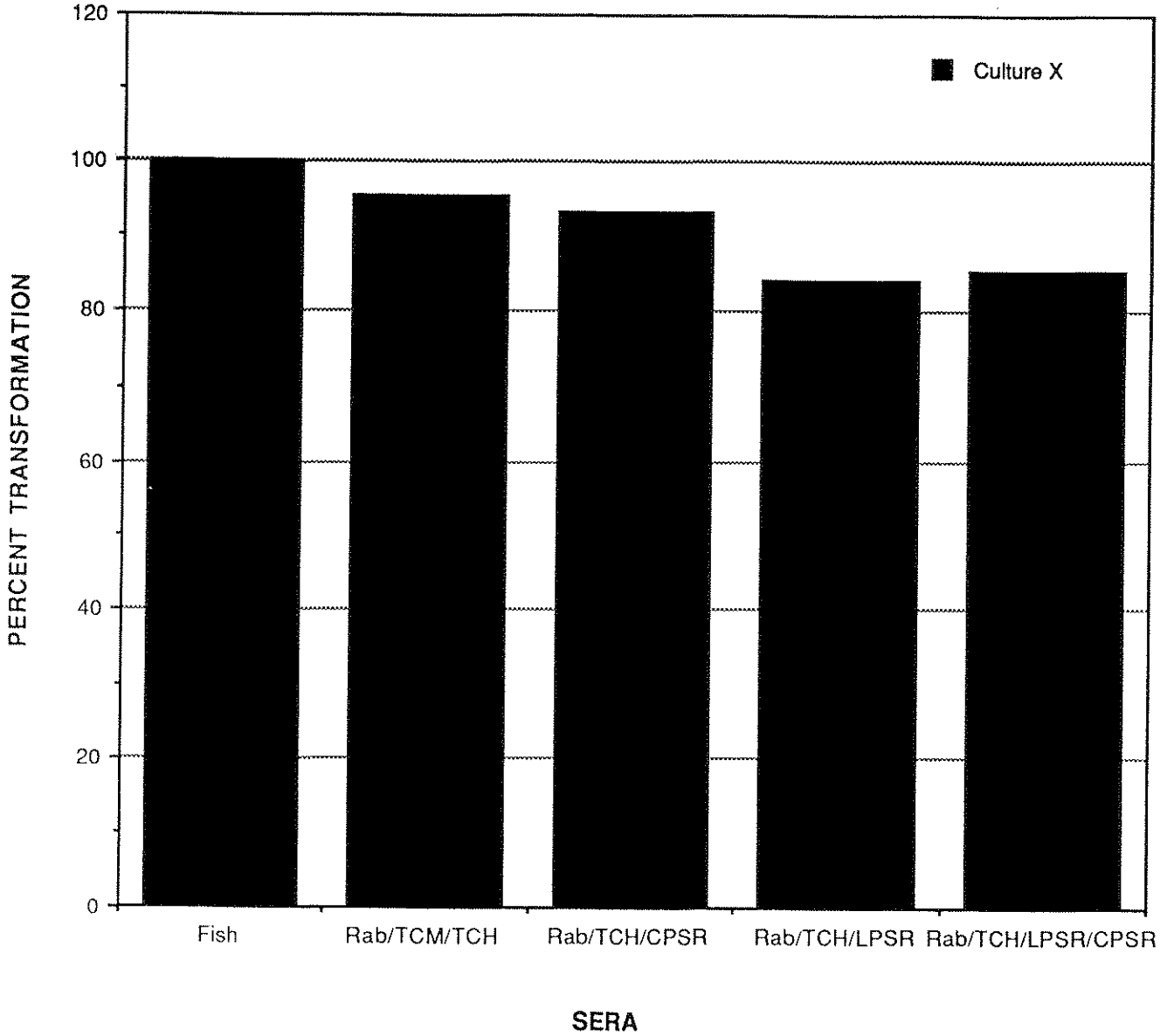
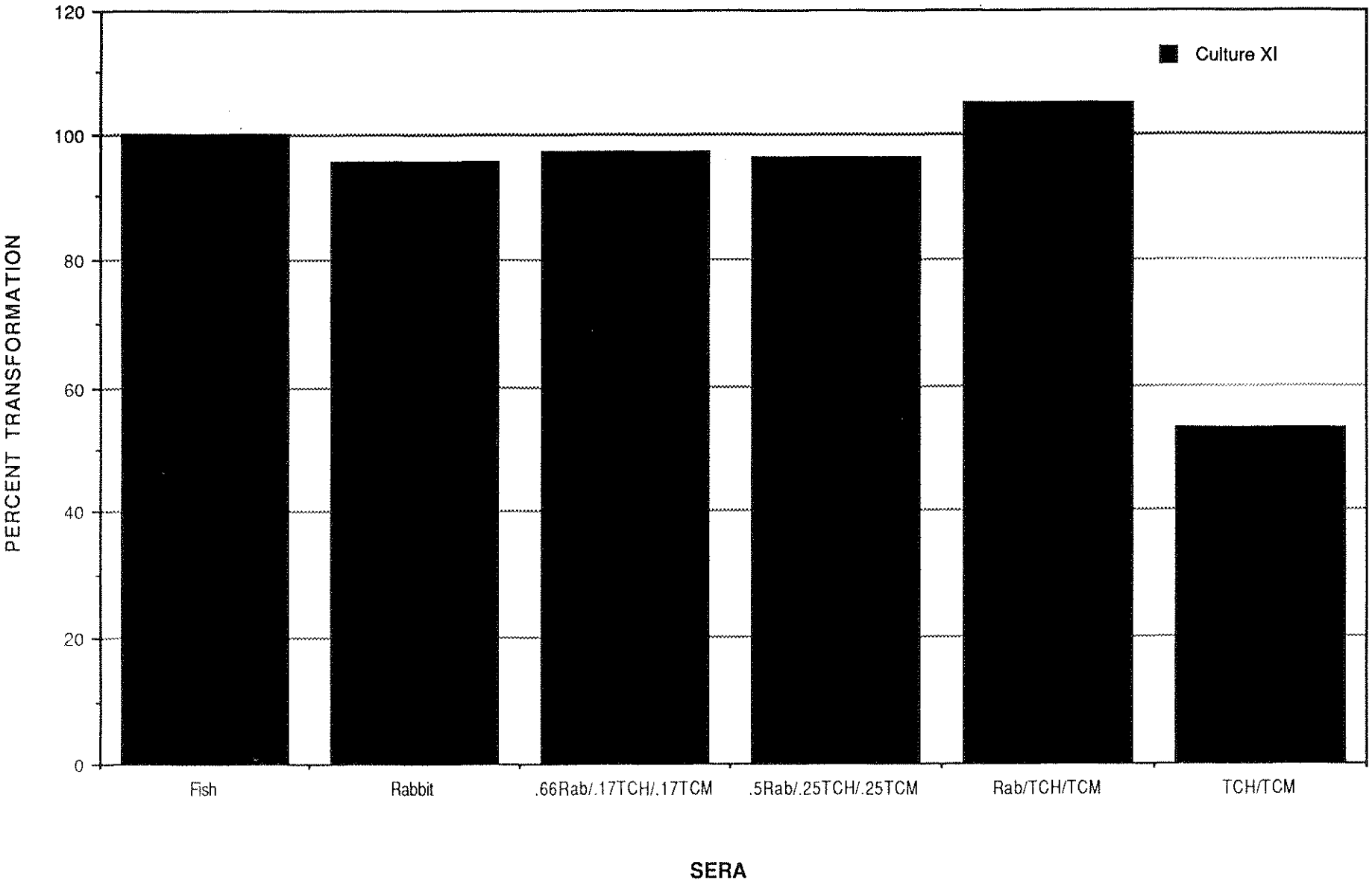


Figure 8. Comparison of serum replacements in combination with rabbit serum in culture XI. (Fish control = 100% transformation, all others relative.)

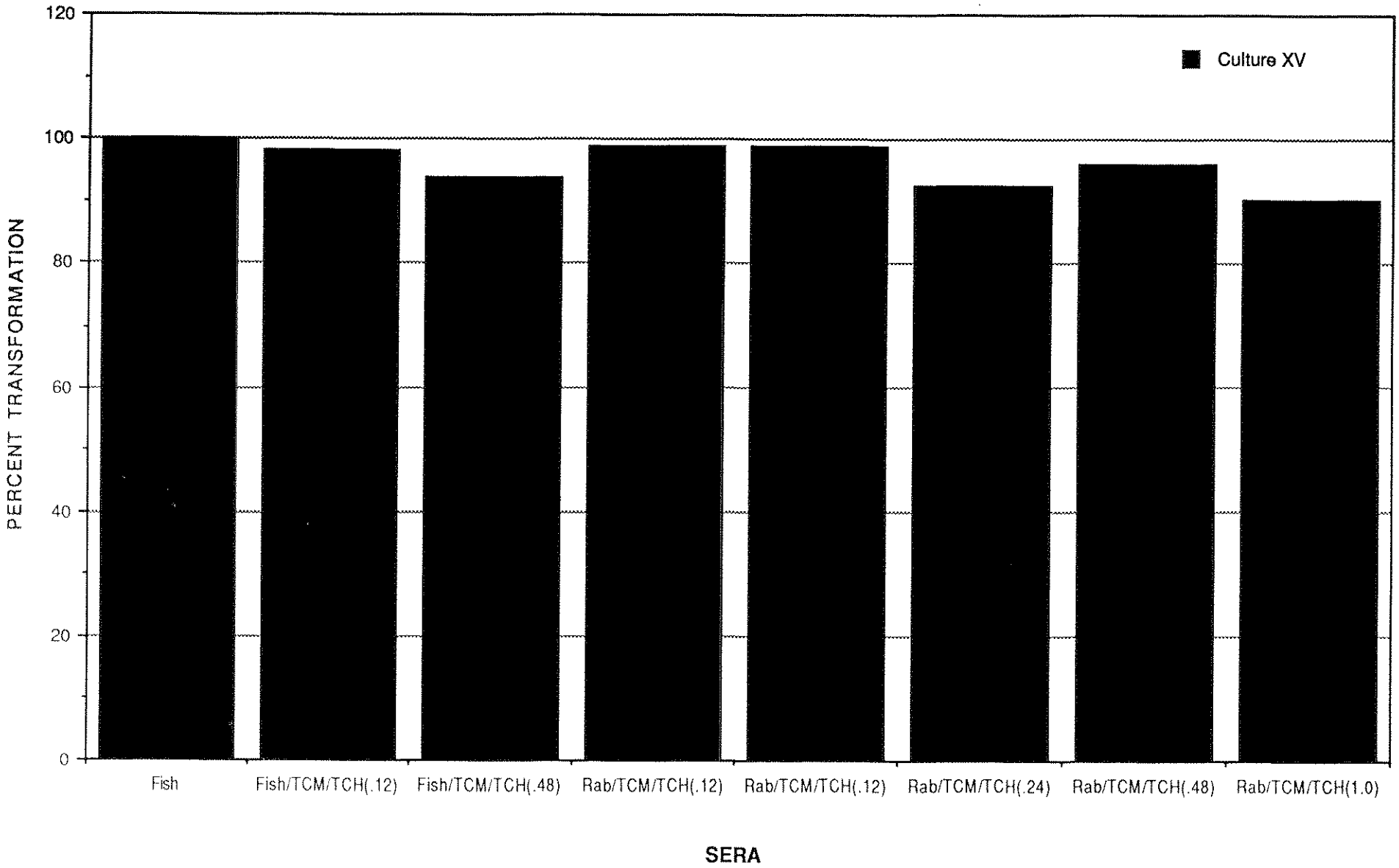


TCH were also mixed with fish plasma at a 1 fish: 1 TCM: 1 TCH ratio. The results indicated that the rabbit mixtures performed as well as those involving fish plasma with the serum replacements (figure 9). Although all performed equally well (chi-square = 5.43, 7 d.f.), the lower concentrations of TCH/TCM appeared to work slightly better in both the fish plasma and rabbit serum. A different ratio of 1.5 rabbit, .75 TCH, .75 TCM using the 12% concentration of the serum replacements yielded almost equal results to the 12% concentration at the usual 1:1:1 ratio (98.9% to 99.0% of fish control yield respectively).

FINAL MEDIUM:

As already discussed, combinations of fish plasma with rabbit serum produced significantly higher results than the fish plasma control (chi-square = 4.1, 1 d.f.); however, our goal was to try to remove the fish plasma from the culture medium for convenience and consistency. The high performance of the 1 rabbit:1 TCH:1 TCM mixture (12% stock of TCH and TCH) was not significantly different from fish control, and did not have any fish plasma in the final medium. This performance suggests that it is the best low serum, no fish plasma, medium for glochidial transformation. By using rabbit serum in equal proportions with TCH and TCM, we have reduced the serum component to 1/3 of its original volume. Additionally, this medium offers more convenience in that the investigator does not have to obtain and process fish blood and greater

Figure 9. Comparison of serum replacements at varying concentrations in combination with fish plasma and rabbit serum in culture XV. (Fish control = 100% transformation, all others relative.)



consistency because of the use of this commercially available serum with processed serum replacements.

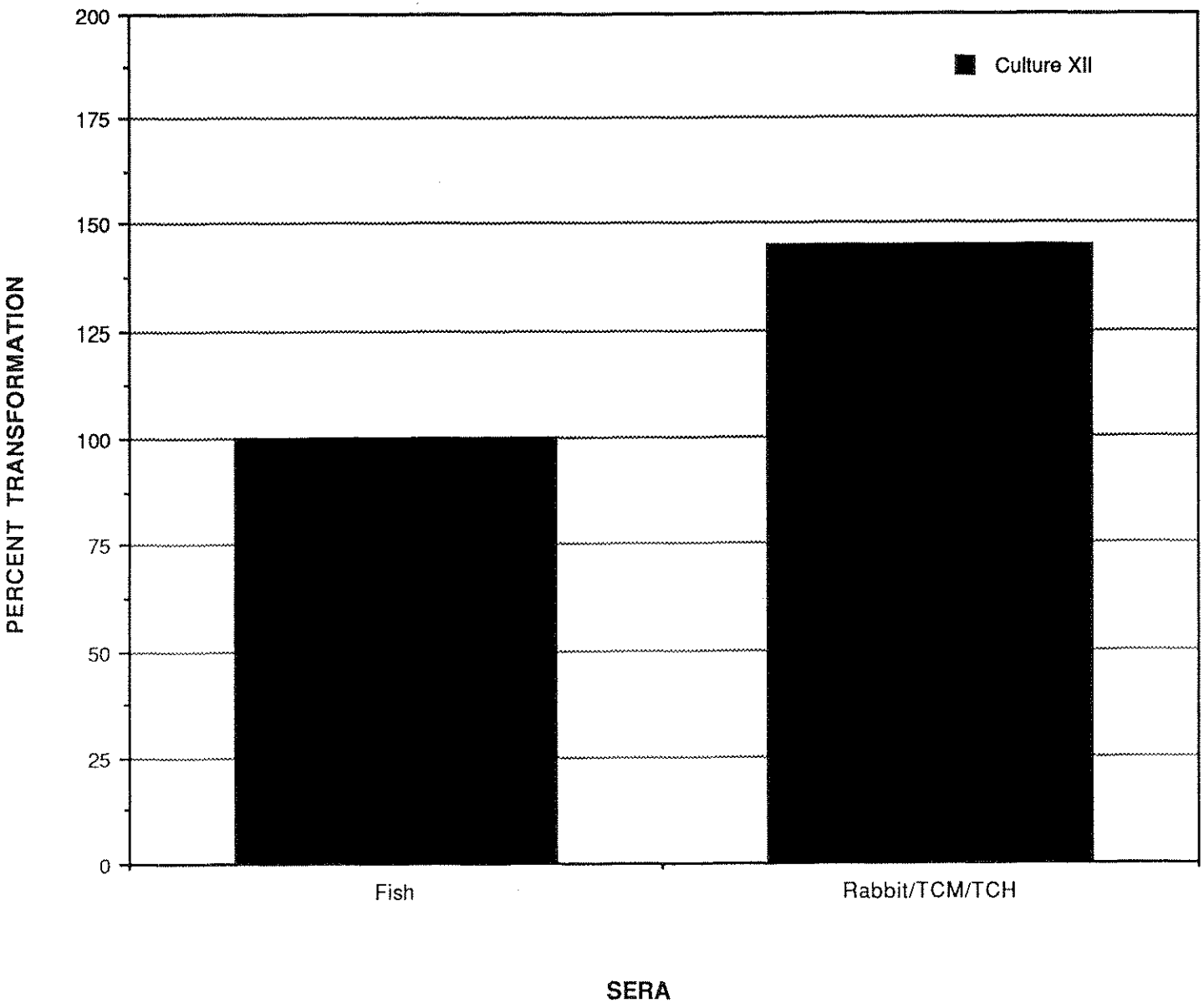
NEW SPECIES:

A culture comparing this latest mixture of rabbit serum, TCH, TCM in Eagle's MEM with the control of fish plasma in MEM control was attempted on a species never before cultured, Elliptio lanceolata. Surprisingly, the new serum produced a significantly higher yield (figure 10) than the fish plasma (chi-square = 17.51, 1 d.f.). This may reflect variations in the fish plasma rather than a species' specific difference; however, Keller and Zam (1990) also had variation in species other than Anodonta imbecilis, which produced a lower (20-40% transformation) rather than a higher yield when their horse serum medium was used. Testing of this rabbit, TCH, TCM combination medium on many more species is necessary in order to predict its consistency of yield on many species.

CONCLUSIONS:

1) Rabbit/TCH/TCM (using 12% stock of TCH and TCM) in a 1:1:1 ratio, when added to Eagle's MEM, produced transformation yields which were equal to fish plasma in Eagle's MEM. The rabbit/TCH/TCM solution was

Figure 10. Response of Elliptio lanceolata to fish plasma medium and rabbit/TCM/TCH medium.



mixed using a ml rabbit serum, 1 ml TCH (12%), 1 ml TCM (12%) and 3 ml of the Eagle's MEM with antibiotics/mycotics as described in Isom and Hudson (1982).

2) The use of commercially available rabbit serum is more convenient and should reduce yield variability when compared to fish plasma yields.

3) The above mixture reduces the use of serum to 1/3 of the original volume of fish plasma (now rabbit serum) by substituting serum replacements for the serum. This should enhance consistency in the culture yields as well as save expense.

4) This improved mixture produced a greater yield than the fish mixture in a culture of the species Elliptio lanceolata; however, cultures of many more species need to be evaluated in order to predict the universality of transformation success.

ACKNOWLEDGEMENTS

The authors of this report are sincerely appreciative to The Tennessee Valley Authority, Presbyterian College and Martin Instrument Company for making funds available to conduct this research. Additional thanks go to Don Wade and Janet Possey of T.V.A. and John Roberts of North Carolina State University for consulting assistance in various phases of this research. Again, thanks go to Don Wade and Janet Possey of T.V.A. and to Ron Dimock of Wake Forest University for providing many of the parent mussels used in the cultures, and finally, to Tim Gaines of Presbyterian College for consultation concerning experimental design and analysis.