Expression and Subcellular Localization of Plasmodium falciparum Aldolase

J. Proiozool. Res., 2. 10-15 (1992)

Expression and Subcellular Localization of Plasmodium falciparum Aldolase

MOTOMI TORII[1], MASAMICHI AIKAWA[1], INDRESH K. SRIVASTAVA[2], MAURICE SCHMIDT[3], KEI-ICHIRO NAKAMURA[1] and LUC H. PERRIN[2]

[1]Department of Pathology, Case Western Reserve University, Cleveland, Ohio, U.S.A.,
[2]Division of Infectious Diseases, and [3]Department of Clinical Chemistry,
Cantonal Hospital, University of Geneva, 1211 Geneva, Switzerland

Received 1 July 1991/ Accepted 29 September 1991

Key words: aldolase, immunoelectron microscopy/ *Plasmodium falciparum*

ABSTRACT
The expression of a *Plasmodium falciparum* 41 kDa blood stage antigen (p41) was followed by examining both protein level and aldolase activity in synchronized cultures. The protein and enzymatic levels were low in erythrocytes containing ring forms, and increased markedly with the appearance of trophozoites and young schizonts. The subcellular localization of p41 aldolase was determined using immunoelectron microscopy with rabbit anti-p41 and anti-recombinant p41 antisera. P41 was localized within the cytoplasm of *P. falciparum* extracellular merozoites, trophozoites and schizonts.

INTRODUCTION
Several defined components of *Plasmodium falciparum* asexual stages may induce partial protection against blood stage infection in immunized monkeys and are therefore considered candidates for the development of malaria vaccines (Hall et al. 1984; Perrin et al. 1985; Collins et al. 1986; Patarroyo et al. 1987). The gene coding for one of these components with a molecular mass of 41kDa (p41) has recently been cloned, and revealed significant homology with known eukaryotic aldolases (Certa et al. 1988; Knapp et al. 1990). Aldolase (D-Fructose-1.6-biphosphate D-glyceraldehyde-3-phosphate lyase, EC 4.1.2.13) acts as a rate limiting step for the degradation of Fructose-1.6-diphosphate (FDP). The importance of this enzyme is reinforced by the fact that *Plasmodium* lack a citric acid cycle and thus have to use high amounts of glucose (WHO scientific group 1987). In the present investigation we have followed the expression of p41 and aldolase activity in synchronized *P. falciparum* cultures and have determined the subcellular localization of p41 by immunoelectron microscopy.

MATERIALS AND METHODS
P. falciparum culture: *P. falciparum* SGE2 isolate was cultivated in 100 x 20 mm petri dishes using the candle jar method (Jensen and Trager 1977; Perrin et al. 1984). Cultures were synchronized by two treatments with 5 % mannitol at an interval of 34 h (Lambros and Vanderberg 1979). The synchronized culture was divided into six 60 x 15 mm petri dishes (5 ml cultures with a hematocrit of 4 %) which were then harvested 4, 12, 20, 28, 36 and 44 h later. The infected red blood cells (IRBC) were washed twice with serum free RPMI 1640 and extracted in 1 ml of lysis buffer (5 mM Tris, 50 mM NaCl, 5 mM EDTA, 1% Nonidet P-40 and 2 mM phenylmethyl sulfonyl fluoride, pH 7.2). The lysate was centrifuged at 60,000 x g for 30 min at 4^{O}C and the supernatant was stored in aliquots at -75^{O}C.

Immunization of rabbits: Purification and analysis of affinity purified and recombinant p41 were carried out as described (Srivastava et al. 1990). Antisera against affinity purified and recombinant p41 were obtained after immunization of rabbits with 100 µg of appropriate antigens in 1 ml of phosphate buffered saline (PBS) mixed with an equal volume of Freund's complete adjuvant (Sigma Chemical Co, USA) followed by two booster immunizations at two week intervals with 100 µg of identically prepared protein in Freund's incomplete adjuvant (Perrin et al. 1985; Srivastava et al. 1990). The anti-p41 rabbit serum immunoprecipitated a single band of 41 kDa from the ^{35}S-methionine labeled combined schizont and merozoite extract and also reacted with p41 in Western blots of up to a dilution of 1/10,000.

Western blot: Five µl of lysate from each of the successive cultures were separated using 9% sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970) and electroblotted on 0.45 µm nitro cellulose paper (Millipore, France) (Dunn 1986). Blots were saturated with 3% bovine serum albumin in PBS (pH 7.4) for 1 h at 37^{O}C, washed once with PBS + 0.1% Tween 20 and incubated with 1/1000 dilution of anti-p41 for 1 h at room temperature. The blots were washed three times for 5 min each with PBS containing 0.1% Tween 20 and incubated with 1/250 dilution of biotinylated anti-rabbit IgG (Amersham, U.K.) for 1 h at room temperature. After three washes, the blots were incubated with 1/400 dilution of Streptavidin-biotinylated horseradish peroxidase complex (Amersham, U.K.) for 30 min at 22^{O}C. The antigen-antibody complex was revealed by the addition of 4 chloro-1 naphthol and H_2O_2 for 10-20 min (22.5 ml PBS + 2.5 ml of 4 mg/ml solution of 4 chloro-1 naphthol + 25 µl of 30% H_2O_2). The reaction was stopped by washing the stripes with distilled water.

Determination of aldolase activity: The aldolase activity present in lysates of successive synchronized cultures was determined using the Test Combination Aldolase reagent kit (UV method Ref. No. 123838) purchased from Boehringer (Mannheim, FRG), and the analysis was performed on a Cobas Fara centrifugal analyzer (Hoffmann-La-Roche, Basel, Switzerland). The aldolase activity assay was based on an estimation of trios phosphate formed from FDP in the presence of limiting quantities of aldolase by convention to α–glycerophosphate dehydrogenase. The rate of the coupled reaction, which reflects the rate of aldolase reaction, was estimated by a spectrophotometric measurement at 340 nm of (5-Nicotinamide adenine dinucleotide oxidation (Beinenherz et al. 1953). One unit of enzymatic activity is defined as the cleavage of 1 µmol of substrate (FDP) per minute.

Aldolase inhibition assay: Anti-p41 was used for the inhibition of aldolase activity on successive cultures of *P. falciparum*. Equal volumes of the successive parasite extracts were incubated with 10 µl of anti-p41 for 5 min at 25^{O}C before starting the assay described above. The percent inhibition was calculated as follows:

$$\%\text{inhibition of aldolase activity} = \frac{\text{Enzyme activity in absence of Ab - Enzyme activity in presence of Ab}}{\text{Total enzyme activity}} \times 100$$

Immunoelectron microscopy: Extracellular merozoites and IRBC were fixed with 1% paraformaldehyde, 0.1% glutaraldehyde in 0.1 M PBS at pH 7.3, and embedded in LR White resin (Polysciences, Inc., Warrington, PA) (Aikawa and Atkinson 1990). Sections were etched with a saturated aqueous solution of sodium metaperiodate for 30 min, blocked for 30 min in 0.1 M PBS containing 5% nonfat dry milk, incubated overnight at 4^{O}C in rabbit anti-p41 or control rabbit serum and then, incubated for 1 h with goat anti-rabbit IgG conjugated to gold particles (Janssen, Piscataway, NJ). Sections were stained with 2% Uranyl acetate in 50% methanol and examined with a JEOL 100CX electron microscope (Torii et al. 1989).

RESULTS AND DISCUSSION

Our results confirm that aldolase activity and protein levels of p41 changed in parallel during the development of *P. falciparum* and reached maxima in the trophozoites and early schizont stages, when

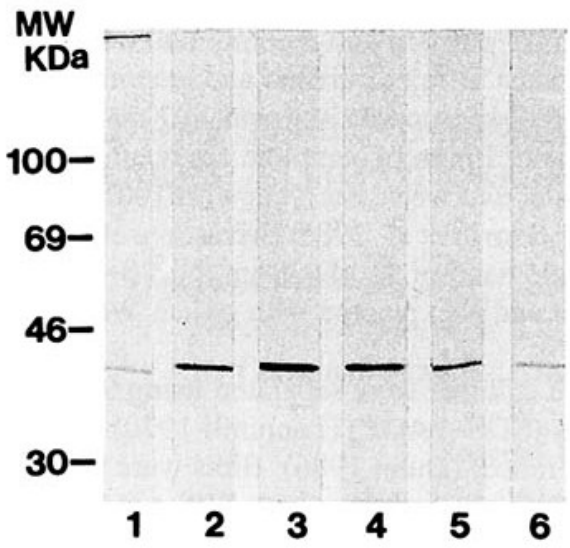

Fig. 1. Expression of p41 in highly synchronized successive *P. falciparum* cultures at 4, 12, 20, 28, 36 and 44 h (Lanes 1 to 6) starting with ring stage (Lane 1) in western blot. Anti-P41 rabbit antiserum was used at a dilution of 1/1000.

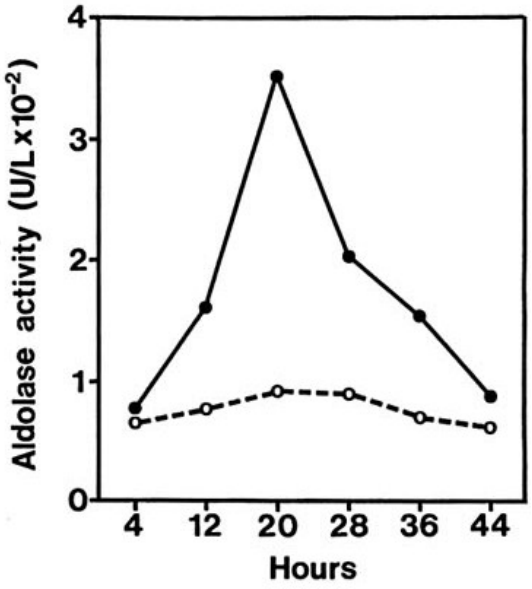

Fig. 2. Parasite aldolase activity during *P. falciparum* asexual blood stage cycle (●—●) and its specific inhibition by anti-p41 rabbit antiserum (○--○).

.

maturation and replication of the parasite occur. The 5 µl lysates from successive cultures containing an identical number of RBC were separated using SDS-PAGE, transferred onto nitrocellulose membranes and probed with rabbit anti-p41 (Fig. 1). Rabbit anti-p41 reacted only with a polypeptide of apparent molecular mass of 41 kDa and no reactivity was observed using an extract of normal RBC (Srivastava et al. 1990). The concentration of p41, as shown by the intensity of the p41 band on the Western blot, was low in preparations of ring forms (fust and last samples) and increased markedly in samples containing trophozoites and schizonts. The percentage of the various blood stages is indicated in Table 1.

These results were confirmed by quantitative measurements of aldolase activity in successive samples (Fig. 2). In cultures of normal RBC the aldolase activity was less than 3 mU/L, corresponding to the activity of human RBC aldolase. The aldolase activity in IRBC containing ring forms was at least twice that observed in normal RBC. The aldolase activity increased markedly with the appearance of trophozoites and young schizonts, but decreased at the time of schizont rupture (appearance of new rings). A markedly reduced level of parasite aldolase activity was measured in the last sample which contained mainly new young ring forms. Here parasite multiplication had increased the parasitemia 2.1 times, compared to the first sample. These results suggest that parasitic aldolase is preferentially synthesized at the trophozoite stage. To confirm that the increase in aldolase

Table 1. Percentage of various asexual blood stages during *P. falciparum* culture

	Time of harvest (hours)					
	4[a]	12	20	28	36	44[b]
Rings	81.0	18.2	4.4	28.5	85.5	94.4
Trophozoites	19.0	81.8	91.2	9.5	7.8	5.6
Schizonts	0	0	4.4	62.0	6.7	0

[a] The parasitemia was 9.5%
[b] Following schizont rupture and reinvasion parasitemia was 20%

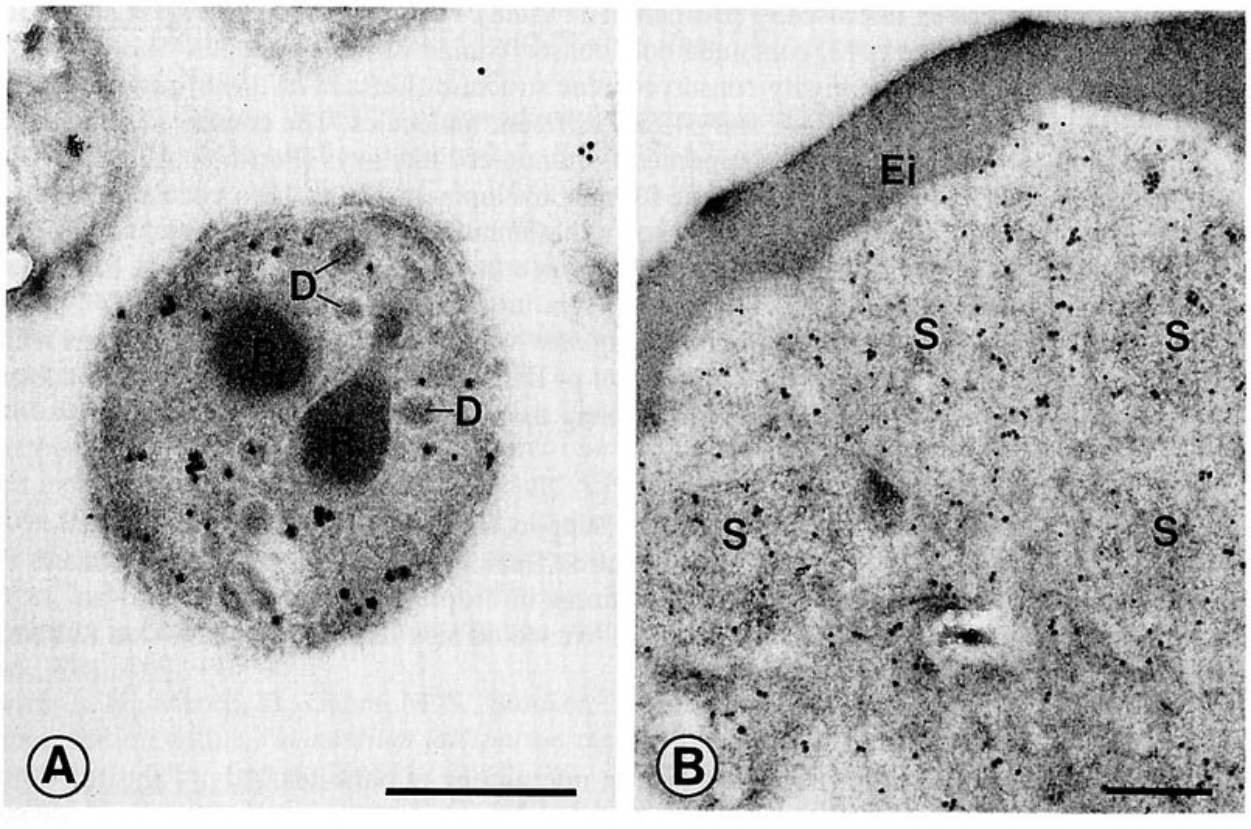

Fig. 3. Immunoelectron microscopic localization of p41 revealed by anti-p41 rabbit serum and colloidal gold. (A) Extracellular merozoites of *P. falciparum* showing localization of gold particles over cytoplasm. Rhoptries (R) and dense granules (D) are not labeled. (B) Young schizonts (S) of *P. falciparum*. Gold particles are scattered over the cytoplasm. The cytoplasms of infected erythrocytes (Ei) are not associated with gold particles. (Bar equals 0.5 μm)

activity was of parasitic origin, successive samples were incubated with rabbit anti-p41 which dramatically depressed parasite aldolase activity (Fig. 2). The remaining activity due to human RBC aldolase was not inhibited by rabbit anti-p41 (Srivastava et al. 1990). Less than 10% inhibition of parasite aldolase activity was observed using normal rabbit antisera or rabbit antisera directed against other *P. falciparum* components (rabbit anti-190-200 kDa, rabbit anti-SHARP, data not shown).

To determine the localization of p41 in the parasite, we performed immunoelectron microscopy using rabbit anti-p41 antiserum and anti-recombinant p41 antiserum. Gold particles revealed a distribution of p41 confined to the cytoplasm, yet not associated with rhoptries, micronemes or dense granules of *P. falciparum* extracellular merozoites (Fig. 3A). In the erythrocytic stage of *P. falciparum*, gold particles were scattered throughout the cytoplasm of trophozoites and schizonts, indicating a diffuse pattern of p41 in these mature parasites. Although cytoplasm of infected erythrocytes remained devoid of gold particles (Fig. 3B). This result is consistent with the finding that aldolase activity is mostly concentrated in the cytosol fractions of the *P. knowlesi* schizonts (Saxena et al. 1986).

Initially, p41 was identified by monoclonal antibodies which reacted not only with 41 kDa but also with 82 kDa components (Perrin and Dayal 1982). The 82 kDa component is processed into a 76 kDa polypeptide which displays protease activity and has been shown to be unrelated to the 41 kDa by pulse-chase experiments (Braun-Breton et al. 1986, 1988). Rhoptry proteins of similar molecular weight (82 kDa and 40/42 kDa) have also been immunoprecipitated by monoclonal antibodies (Schofield et al.

1986, Clark et al. 1987). These monoclonal antibodies react by immunofluorescence with rhoptries of merozoites and diffusely within whole schizonts. Using a monoclonal antibody which reacts only with a 42 kDa component (p42), p42 was proven to be localized within the Rhoptry organelle by immunoelectron microscopy (Bushell et al. 1988). Furthermore, Schofield et al. (1986) reported that the target antigen (p42) contained both conserved and variable epitopes. These observations are in contrast to the highly conserved gene structure (Certa ct al. 1988) and subcellular localization of p41, and suggest that p41 and p42 are different molecules. The conserved structure of p41 and its capacity to induce protective immunity in immunized monkeys (Perrin et al. 1985) suggests that this molecule might be a candidate for the development of a malaria vaccine. Despite previous reports, however, it has been recently shown that immunization with recombinant p41 did not protect monkeys from a challenging infection (Herrera et al. 1990). Recombinant p41 lacks the first five amino acids of the natural p41. The monkey sera immunized with natural p41 and challenged with *P. falciparum* contained antibodies against several additional parasite antigens which were missing in the sera immunized with recombinant p41 (Herrera et al. 1990). These differences could account for the discrepancies that appeared among these reports.

ACKNOWLEDGEMENTS

We would like to gratefully acknowledge the support from the Agency for International Development (DPE-0453-00-4027-00), The US Public Health Service (AI-10645), the United Nations Development Program for Research and Training in Tropical Medicine and the Swiss National Research Foundation Grant (31-26508.89). We would also like to thank Kiet Dan Luc for his excellent technical assistance.

REFERENCES

Aikawa, M. & Atkinson, C. T. 1990. Immunoelectron microscopy of parasites. *Adv. Parasitol. 29*: 151-214.

Beinenherz, G., Boltze, H. J., Bticker, T., Czok, R., Garbade, E., Meyer-Arendt, E. & Pfleiderer, G. 1953. Diphosphofructose-Aldorase; Phosphoglyceraldehyde-Dehydrogenase, Milchsäure- Dehydrogenase; Glycerophosphate Dehydrogenase und Pyruvate kinase auf Kanrinchenmuskelatur in einem Arbeitsgang. *Z. Naturforsch. 86*: 555-577.

Braun-Breton, C, Jendoubi, M., Brunet, E., Perrin, L., Scaife, J. & Pereira da Silva, L. 1986. In vitro time course of synthesis and processing of major schizont membrane polypeptides in *Plasmodium falciparum*. *Mol. Biochem. Parasitol. 20*: 33-43.

Braun-Breton, C., Rosenberry, T. L. & Pereira da Silva, L. 1988. Induction of the proteolytic activity of a membrane protein in *Plasmodium falciparum* by phosphatidyl inositol-specific phospholipase C. *Nature 332*: 457-459.

Bushell, G. R., Ingram, L. T, Fardoulys, C. A. & Cooper, J. A. 1988. An antigenic complex in the rhoptrics of *Plasmodium falciparum*. *Mol. Biochem. Parasitol. 28*: 105-112.

Certa, U., Ghersa, P., Döbeli, H., Matile, H., Kocher, H. P., Srivastava, I. K., Shaw, A. R. & Perrin, L. H. 1988. Aldolase activity of a *Plasmodium falciparum* protein with protective properties. *Science 240*: 1036-1038.

Clark, J. T., Anand, R., Akoglu. T. & McBride, J. S. 1987. Identification and characterization of proteins associated with the rhoptry organdies of *Plasmodium falciparum* merozoites. *Parasitol. Res. 73*: 425-434.

Collins, W. E., Anders, R. F., Pappaioanou, M., Campbell, G. H., Brown, G. V., Kemp, D. J., Coppel, R. L., Skinner, J. C., Andrysiak, P. M., Favaloro, J. M., Corcoran, L. M., Broderson, J. R., Mitchell, G. F. & Campbell, C. C. 1986. Immunization of *Aotus* monkeys with recombinant proteins of an erythrocyte surface antigen of *Plasmodium falciparum*. *Nature 323*: 259-262.

Dunn, S. D. 1986. Effects of the modification of transfer buffer composition and the renatutation of proteins in gels on the recognition of proteins on western blots by monoclonal antibodies. *Anal. Biochem. 157*: 144-153.

Hall, R., Hyde, J. E., Goman, M., Simmons, D. L., Hope, I. A., Mackay, M., Scaife, J., Merkli, B., Richle, R. & Stocker, J. 1984. Major surface antigen gene of a human malaria parasite cloned and expressed in bacteria. *Nature 311*: 379-382.

Herrera, S., Herrera, M. A., Perlaza, B. L., Burki, Y., Caspers, P., Döbeli, H., Rotmann, D. & Certa, U. 1990. Immunization of *Aotus* monkeys with *Plasmodium falciparum* blood-stage recombinant proteins. *Proc. Natul. Acad. Sci. 87*: 4017-4021.

Jensen, J. B. & Trager, W. 1977. *Plasmodium falciparum* in culture: use of outdated erythrocytes and description of the candle jar method. *J. Parasitol. 63*: 883-886.

Knapp, B., Hundt, E. & Küpper, H. A. 1990. *Plasmodium falciparum* aldolase: gene structure and localization. *Mol. Biochem. Parasitol. 40*: 1-12.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature 227*: 680-682.

Lambros, C. & Vanderberg, J. P. 1979. Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. *J. Parasitol. 65*: 418-420.

Patarroyo, M. E., Romero, P., Torres, M. L., Clavijo, P., Moreno, A., Martinez, A., Rodriguez, R., Guzman, F. & Cabezas, E. 1987. Induction of protective immunity against experimental infection with malaria using synthetic peptides. *Nature 328*: 629-632.

Perrin, L. H. & Dayal, R. 1982. Immunity to asexual erythrocytic stages of *Plasmodium falciparum*: role of defined antigens in the humoral response. *Immunol. Rev. 61*: 245-269.

Perrin, L. H., Merkli, B., Loche, M., Chizzolini, C., Smart, J. & Richle, R. 1984. Antimalarial immunity in Saimiri monkeys immunization with surface components of asexual blood stages. *J. Exp. Med. 160*: 441-451.

Perrin, L. H., Merkli, B., Gabra, M. S., Stocker, J. W., Chizzolini, C. & Richle, R. 1985. Immunization with a *Plasmodium falciparum* merozoite surface antigen induces a partial immunity in monkeys. *J. Clin. Invest. 75*: 1718-1721.

Saxena, N., Pandey, V. C., Dutta, P. G. & Ghatak, S. 1986. Level of FDP-aldolase enzyme activity in *Plasmodium knowlesi* parasitized monkey erythrocytes. *Indian. J. Exp. Biol. 24*: 502-504.

Schofield, L., Bushell, G. R., Cooper, J. A., Saul, A. J., Upcroft, J. A. & Kidson, C. 1986. A rhoptry antigen of *Plasmodium falciparum* contains conserved and variable epitopes recognized by inhibitory monoclonal antibodies. *Mol. Biochem. Parasitol. 18*: 183-195.

Srivastava, I. K., Schmidt, M., Certa. U., Döbeli, H. & Perrin, L. H. 1990. Specificity and inhibitory activity of antibodies to *Plasmodium falciparum* aldolase. *J. Immunol. 144*: 1497-1503.

Torii, M., Adams, J. H., Miller, L. H. & Aikawa, M. 1989. Release of merozoite dense granules during erythrocyte invasion by *Plasmodium knowlesi*. *Infect. Immun. 57*: 3230-3233.

WHO Scientific Group. 1987. The biology of malaria parasites. *WHO Tech. Rep. Scr. 1987* No. 743.