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Phosphoprotein Patterns in *Onchocerca Volvulus* Developmental Stages

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ABSTRACT

Living females and microfilariae of *Onchocerca volvulus* incubated in culture medium containing [^{32}P]orthophosphate were observed to phosphorylate their proteins rapidly. Patterns of phosphoproteins in extracts from these labelled parasites were compared after two dimensional electrophoresis and autoradiography. Protein extracts from eggs, microfilariae and adult females of *O. volvulus* were phosphorylated in the presence of [γ - ^{32}P]ATP, magnesium acetate, and added cyclic AMP - dependent protein kinase or the endogenous protein kinase present in the extracts. Patterns of phosphoproteins were compared after separation by single and two - dimensional gel electrophoresis followed by autoradiography. Common phosphopeptide bands were observed when phosphorylated extracts from adult females, microfilariae and eggs were compared. However, extracts from eggs displayed unique phosphorylated polypeptides of M_r 30 000 and 34 000 that were absent from the extracts from microfilariae. Furthermore, two phosphorylated polypeptides of M_r 47 000 and 76 000 were detected in extracts from microfilariae but not from eggs. These results indicate that *O. volvulus* parasites may phosphorylate different proteins at different stages of their development.

INTRODUCTION

Onchocerca volvulus is a filarial parasite that infests about 17 million people living in the endemic tropical countries with a risk population of about 85 million (22). Onchocerciasis, the disease caused by this parasite, results in severe pruritus, dermatitis, subcutaneous nodules and eye involvement leading to blindness. Consequently, considerable effort has been employed to develop new and effective control measures for the disease (22). This notwithstanding, large scale eradication of onchocerciasis is not yet feasible. The microfilaricide ivermectin, at the normal dose regimen,

does not kill adult *O. volvulus* which are able to resume reproduction once treatment has stopped (5), thereby initiating a recrudescence of the symptoms associated with microfilaridermia.

It has been recommended to investigate the parasite's metabolism with the hope of finding new targets for rational drug development (21). Up to now, only a few studies have dealt with the metabolism of *O. volvulus* and its control (16 - 19). However, protein phosphorylation has attracted our attention since the partially purified protein kinase of *O. volvulus* has been shown to be inhibited by suramin, a potent but relatively toxic adulticide (19). Protein phosphorylation is known to modulate the function of several key proteins (8). Protein kinase substrates were demonstrated in *O. volvulus* extracts phosphorylated in vitro, but their function remains unknown (17). In order to gain further insight into their importance, these phosphoproteins have been compared in the developmental stages of *O. volvulus*.

MATERIALS AND METHODS

Reagents: [γ - ^{32}P]ATP was purchased from New England Nuclear, [^{32}P]orthophosphate was from Amersham. Culture media were from GIBCO and Flow Laboratories. Fetal calf serum was from Flow Laboratories. Ampholines, molecular weight marker proteins, protein A Sepharose CL - 4B and Sephadex G - 50 were products of Pharmacia LKB Biotechnology. All other biochemicals and reagents were obtained from Sigma, Merck, Serva and BDH. The catalytic subunit of cyclic AMP - dependent protein kinase (cAPK) was purified from rabbit muscle as described previously by Betchel and coworkers (1).

Parasites: *O. volvulus* adult females (4, 20), microfilariae and eggs (15) were isolated and checked for viability as described. The parasites were kept at 28 - 30°C in RPMI - 1640 (GIBCO) supplemented with 10% fetal calf serum and 0.16 mg/ml gentamycine until used for the experiments within 24 h.

In situ phosphorylation: Intact viable females of *O. volvulus* were preincubated for 1 h in phosphate - free minimal essential medium (Flow Laboratories) supplemented with 10% fetal calf serum, 2 mM glutamine and 0.16 mg/ml gentamycine. Carrier - free [^{32}P]orthophosphate (0.2 mCi) was added to the medium and the incubation was continued for 2 h. The labelled parasites were washed five times with 5 ml of phosphate buffered saline (PBS) and were then homogenized in a Potter Elvehjem homogenizer in 1 ml of 50 mM phosphate buffer pH 7.6 containing 100 mM NaF, 5 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulphonyl fluoride (PMSF) and 1% (w/v) Nonidet P40. After centrifugation of the homogenate using a speed of 25 000 x g for 20 min at 4°C, the supernatant was collected, precipitated with cold acetone at - 20°C, dissolved in lysis buffer and analyzed by two - dimensional gel electrophoresis (IEF/SDS - PAGE).

About 2 x 10^5 microfilariae of *O. volvulus* were preincubated in phosphate - free minimal essential medium, supplemented as described in the previous paragraph. After the preincubation, 0.3 mCi of carrier - free [^{32}P]orthophosphate was added and the parasites were incubated overnight at room temperature with gentle shaking. Labelling was stopped by adding 5 ml of PBS to the parasites and centrifuging at 2 500 x g for 5 min. The supernatant was sucked off and the parasites were resuspended in 5 ml of PBS. This washing procedure was repeated four times after which proteins were extracted from the parasites by sonication for 30 s bursts in 10 mM phosphate buffer pH 7.4 containing 0.25 M sucrose, 1 mM EDTA, 1 mM diisopropyl fluorophosphate (DFP) and 1 mM DTT. Centrifugation was done at 25 000 x g for 20 min at 4°C after which the supernatant was collected and analyzed by IEF/SDS - PAGE.

Preparation of protein extracts: Microfilariae and eggs for extraction were centrifuged at 2 000 x g for 10 min and resuspended in PBS. The washing cycles of centrifugation and resuspension were repeated five times. Adult worms were simply resuspended in 5 ml of PBS and allowed to stand for five min. The PBS was then withdrawn with a sterile Pasteur pipette.

Proteins were extracted from adult worms as previously described (17), concentrated to 2 mg/ml and stored in aliquots of 0.5 ml at - 70°C for subsequent use in phosphorylation experiments. About 6 x 10^5 eggs and 1.5 x 10^5 microfilariae were extracted by sonication in 1 ml of 10 mM phosphate buffer pH 7.4, 0.25 M sucrose, 1 mM EDTA, and 1 mM dithiothreitol (DTT) using an MSE Soniprep, set at an amplitude of 10 microns. After microscopic confirmation of parasite disintegration, the sonicated material was centrifuged at 25 000 x g for 20 min at 4°C. The supernatants were collected and stored as above until used.

Phosphorylation of worm extracts: Phosphorylation of *O. volvulus* protein extracts was carried out in a final volume of 50 μl containing 10 mM magnesium acetate, 40 mM phosphate buffer pH 6.4, 50 μg of *O. volvulus* protein, and 1 mM [γ - ^{32}P]ATP (specific activity 10 - 20 cpm/pmol). Where present, cyclic adenosine 3':5' monophosphate (cAMP) and the cAPK were used at final concentrations of 10^{-4} M and 20 mg/ml, respectively. Incubations were carried out at 30°C for 10 min. For samples analyzed with polyacrylamide gel electrophoresis in sodium dodecyl sulphate (SDS - PAGE), the reaction was stopped by the addition of the sample buffer described by O'Farrell (11).

Protein extracts phosphorylated for IEF/SDS - PAGE were incubated as above but the specific activity of [γ - ^{32}P]ATP was raised to about 100 cpm/pmol. The reaction was stopped by adding two volumes of 1.5 times concentrated lysis buffer (11).

Immunoprecipitations: [^{32}P]phosphoproteins in 50 μl of the reaction mixture with adult worm extracts, in the absence of added cAMP and cAPK, were incubated with normal European serum or

a pool of Cameroonian onchocerciasis sera diluted 10 - fold. Immunoprecipitations were carried out according to the method of Kessler (7) with modifications. The immune complexes formed were isolated by using protein A Sepharose CL - 4B and analyzed by SDS - PAGE followed by autoradiography (14).

Electrophoresis: SDS- PAGE was performed in 11% slab gels using the buffer system of Laemmli (10). Aliquots of the phosphorylation mixture containing 25 μg of protein, and mixed with sample buffer as described above, were treated by heating at 90°C for 3 min, applied to the gel and electrophoresed at 90 volts for 12 - 14 h. After fixing, the gels were autoradiographed at -70°C for 3 - 7 days.

In other experiments, phosphorylated *O. volvulus* protein extracts (ca 5 000 cpm), mixed with lysis buffer as described above, were analyzed by use of IEF/SDS - PAGE, essentially as described by O'Farrell (11)

Protein was determined according to the method of Read and Northcote (12) with bovine serum albumin as a standard.

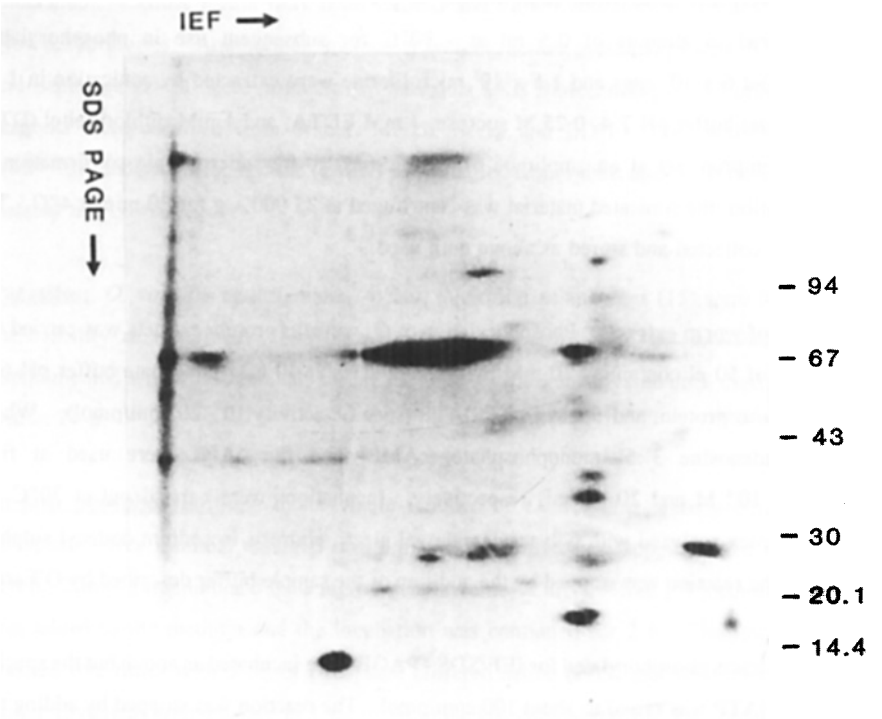

Fig. 1. Patterns of phosphoproteins from [^{32}P]orthophosphate - labelled adult females of *O. volvulus*. Analyses were with performed IEF/SDS - PAGE as described under "Materials and Methods". M_r x 10^{-3} are indicated.

RESULTS

Protein phosphorylation in intact females and microfilariae of *O. volvulus*: In order to determine whether protein phosphorylation occurs in viable *O. volvulus* parasites under physiological conditions, intact living females and microfilariae of *O. volvulus* were labelled with [^{32}P]orthophosphate. Fig. 1 shows an autoradiograph obtained by analyzing proteins from living adult females labelled with [^{32}P]orthophosphate using IEF/SDS - PAGE. At least 30 spots corresponding to phosphorylated polypeptides with M_r ranging from 14 000 to 180 000 could be observed.

Proteins extracted from [^{32}P]orthophosphate - labelled microfilariae were also analyzed by IEF/SDS - PAGE and autoradiography (Fig. 2). At least 20 spots corresponding to phosphorylated polypeptides were usually observed. Most of the heavily labelled polypeptides were usually found in the low molecular weight range.

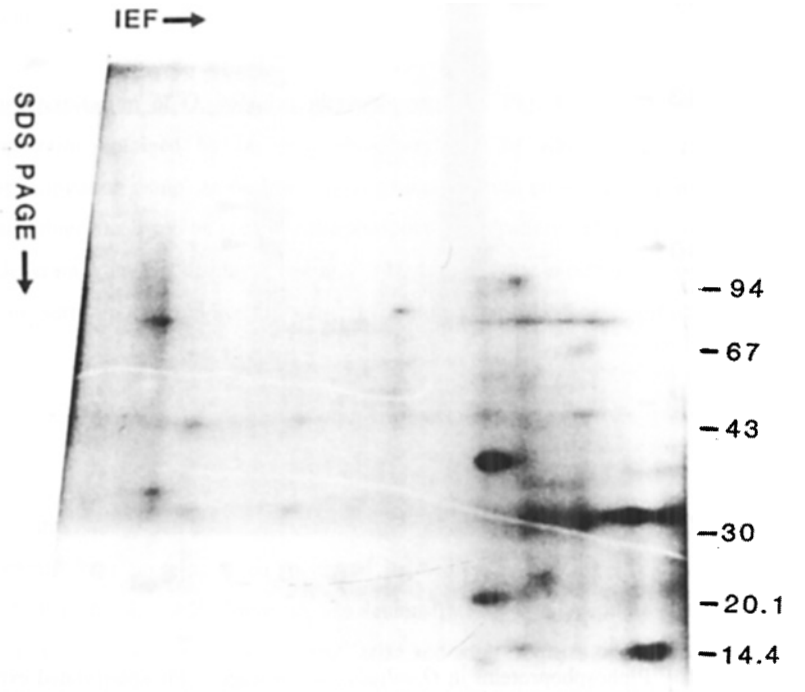

Fig. 2. Patterns of phosphoproteins from [^{32}P]phosphate - labelled viable *O. volvulus* microfilariae. The proteins from microfilariae labelled as described under "Materials and Methods", were analyzed by IEF/SDS - PAGE followed by autoradiography. M_r x 10^{-3} are indicated.

Patterns of phosphoproteins in extracts from *O. volvulus* worm stages: After showing that intact viable parasites were capable of protein phosphorylation, we carried out phosphorylation of extracts in order to gain an insight into the nature of the protein kinases involved. The reaction conditions for the phosphorylation were established by carrying out preliminary experiments in which protein extracts (25 μg) from adult females of *O. volvulus* were phosphorylated in buffer ranging from pH 6.4 to pH 8.0. It was observed (results not illustrated) that maximal incorporation of phosphate into the protein occurred at pH 6.4 which was selected for subsequent phosphorylations.

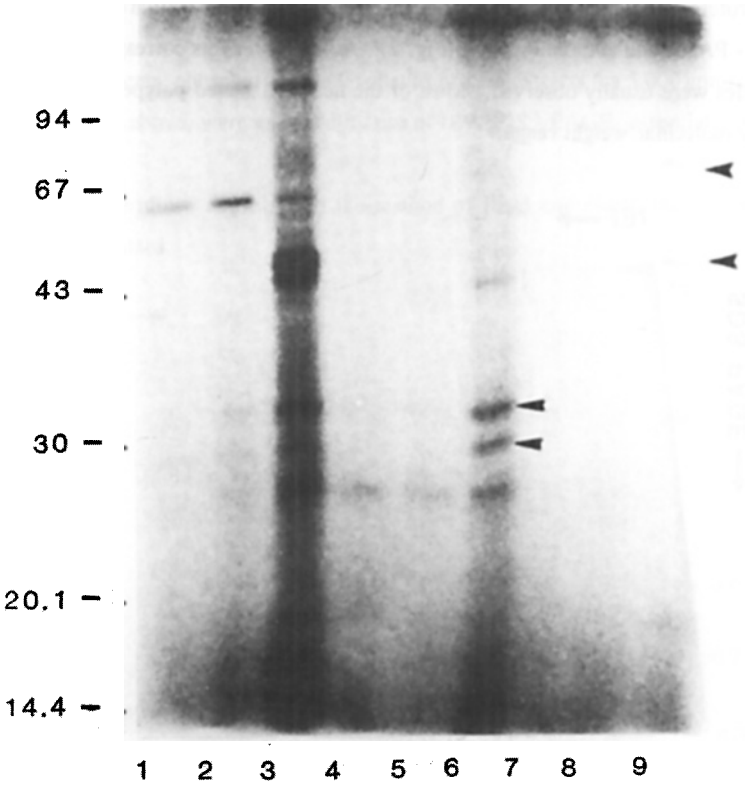

Fig 3: Patterns of [^{32}P]phosphoproteins in *O.volvulus* worm stages. Phosphorylated extracts from each worm stage (7.5 μg) were analyzed by SDS - PAGE and autoradiography. Lanes 1, 2 and 3: Phosphoproteins from adult females obtained in the presence of [γ - ^{32}P]ATP alone (1), [γ - ^{32}P]ATP + cAMP (2) and [γ - ^{32}P]ATP + cAPK (3). Lanes 4, 5 and 6: Phosphoproteins from eggs obtained in the presence of [γ - ^{32}P]ATP alone (4), [γ - 32P]ATP + cAMP (5) and [γ - ^{32}P]ATP + cAPK (6). Lanes 7, 8 and 9: Phosphoproteins from microfilariae obtained in the presence of [γ - ^{32}P]ATP alone (7), [γ - 32P]ATP + cAMP (8) and [γ - ^{32}P]ATP + cAPK (9). Arrows indicate stage - specific phosphoproteins observed in eggs and microfilariae. Location of the molecular weight marker proteins is indicated as M_r x 10^{-3}.

The phosphoprotein patterns obtained with extracts from eggs, microfilariae and adult females of *O. volvulus* were compared after electrophoresis and autoradiography. The autoradiograph (Fig. 3) reveals that in the absence of exogenous cAPK, the extracts from eggs and microfilariae were phosphorylated only to a limited extent. On the contrary, extracts from adult females showed at least 5 phosphoprotein bands. Upon addition of rabbit muscle cAPK, the phosphorylation of proteins from eggs and microfilariae was enhanced (lanes 6 & 9). In extracts from eggs, at least two major specific polypeptide bands of M_r 30 000 and 34 000 were observed (lane 6). In microfilariae, bands of phosphorylated polypeptides of M_r 47 000 and 76 000 were present(lane 9); however, they were absent in extracts from eggs. Analyses of phosphorylated extracts from eggs and microfilariae by IEF/SDS - PAGE revealed that these apparently stage - specific bands could be observed as distinct spots on the autoradiographs. When protein extracts from adult females were phosphorylated and analyzed by IEF/SDS - PAGE, at least 25 spots could be observed on the autoradiographs. Some of these spots had apparent molecular weights similar to those found in eggs and microfilariae (results not shown).

Immunoprecipitation of *O. volvulus* phosphoproteins: Fig. 4 shows an autoradiograph of the phosphoproteins obtained by in vitro phosphorylation of adult worm extracts followed by immunoprecipitation using an onchocerciasis patients' serum pool and normal human serum (from European subjects). It can be seen that the phosphoprotein pattern recognized by the immune serum pool is different from that displayed in Fig. 3. Under the same experimental conditions, the control (European) serum pool from healthy subjects did not precipitate phosphoproteins to any significant extent.

DISCUSSION

In this study we have provided evidence to the effect that living *O. volvulus* parasites phosphorylate their proteins when incubated in medium containing [^{32}P]orthophosphate. Using [γ - ^{32}P]ATP as the phosphate donor we also demonstrated that polypeptides could be phosphorylated in protein extracts made from eggs, microfilariae and adult females of *O. volvulus* using either the endogenous protein kinase(s) or added cAPK. Some of the polypeptide bands phosphorylated in adult females had similar relative molecular weights to those phosphorylated in eggs and microfilariae. This can be explained, in part, by the fact that gravid adult females of *O. volvulus* usually contain both microfilariae and eggs in their uteri. However, when patterns of phosphorylated polypeptides from eggs and microfilariae were compared, each of the worm stages revealed a distinct set of phosphorylated polypeptides, thus indicating that the phosphoprotein patterns may vary during the development of the parasite. When patterns of phosphorylated polypeptides obtained from microfilariae labelled with [^{32}P]orthophosphate in situ were compared with those in extracts from the

same parasite stage labelled with [γ - ^{32}P]ATP, we noticed that the number of polypeptides phosphorylated in situ is greater than the number obtained in extracts in the presence of a high concentration of highly purified cAPK from rabbit muscle. This difference probably reflects the optimal localization of the relevant protein kinase(s) and substrates in living parasites.

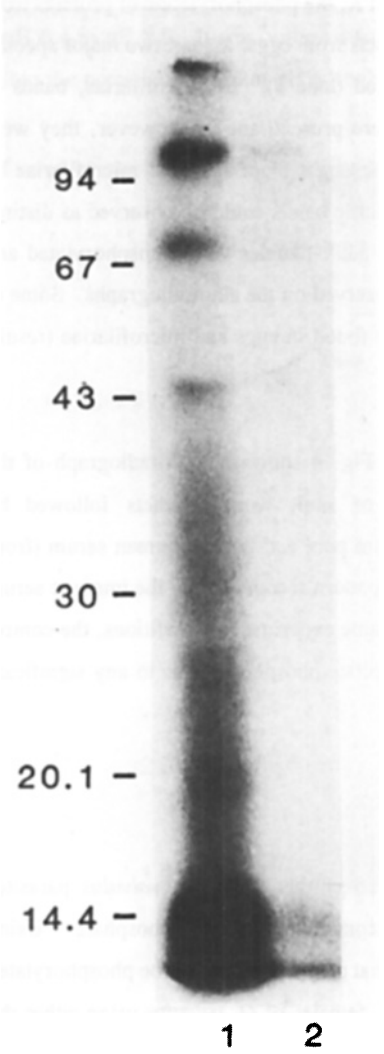

Fig. 4. Patterns of immunoprecipitated phosphoproteins of adult worm extracts using immune serum from onchocerciasis patients. Analysis was performed by SDS -PAGE. Lane 1, phosphoproteins recognized by onchocerciasis infection serum; lane 2, phosphoproteins recognized by the control (European) serum. $M_r \times 10^{-3}$ are indicated.

In some of the in vitro experiments, the extracts were enriched with high concentrations of highly purified catalytic subunit of rabbit skeletal muscle cAPK in order to counterbalance the action of phosphoprotein phosphatases. The essentially pure and highly active enzyme of mammalian origin was preferred to a partially purified and therefore less active preparation of *O. volvulus* protein kinase(s). This measure was also considered to minimize carry - over of phosphorylatable proteins that would exist in partially purified *O. volvulus* protein kinase(s). Furthermore, the catalytic subunit

of cAPK is highly conserved among eukaryotic organisms (6), and the specificity of cAPK is defined by a short sequence of amino acids in several substrates (23). Therefore, proteins phosphorylated by exogenous cAPK may be tentatively considered as substrates of the protein kinase in *O. volvulus*.

In our experiments, an immune serum pool from onchocerciasis patients was shown to precipitate some of the phosphoproteins of *O. volvulus* extracts (Fig. 4), suggesting that these phosphoproteins are antigens involved in the host - parasite interactions. It is not known whether the host response to these proteins varies with their phosphorylation state.

Experiments with other nematodes have shown that phosphorylation/dephosphorylation may be implicated in the regulation of the glycolytic enzyme phosphofructokinase (2, 3, 9, 13). The moderate quantities of *O. volvulus* material used for the present work precluded the purification and identification of protein kinase substrates detected. In this study we have shown that protein phosphorylation occurs in *O. volvulus* under physiological conditions. It would be necessary to functionally characterize the major phosphoproteins of *O. volvulus*. If their phosphorylation is shown to be involved in regulation of parasite metabolism, then such phosphorylation events may constitute a target for developing new chemotherapeutic agents against onchocerciasis.

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REFERENCES

1) Betchel, P. J., Beavo, J. A. & Krebs, E. G.: Purification and characterization of the catalytic subunit of skeletal muscle adenosine 3':5' monophosphate - dependent protein kinase. **J Biol Chem 252:** 2691 - 2697, 1977.

2) Daum, G., Thalhofer, H. P., Harris, B. G., & Hofer, H. W.: Reversible activation and inactivation of phosphofructokinase from *Ascaris suum* by the activation of tissue homologous protein phosphorylating and dephosphorylating enzymes. **Biochem Biophys Res Comm 139:** 215 - 221, 1986.

3) Daum, G., Schmid, C., MacKintosh, C., Cohen, P., & Hofer, H. W.: Characterization of the major phosphofructokinase dephosphorylating protein phosphatases from *Ascaris suum* muscle. **Biochim Biophys Acta 1122:** 23 - 32, 1992.

4) Engelbrecht, F. & Schulz - Key, H.: Observations on adult *Onchocerca volvulus* maintained

in vitro. **Trans R Soc Trop Med Hyg 78:** 212 - 215, 1984.

5) Greene, B. M., Brown, K. R. & Taylor, H. R.: Use of Ivermectin in humans. In: **Ivermectin and Abamectin** (ed. W. C. Campbell), pp. 311 - 323. Springer - Verlag, New York, 1989.

6) Hanks, S. K., Quinn, A. M. & Hunter, T.: The Protein Kinase Family: Conserved Features and Deduced Phylogeny of the Catalytic Domains. **Science 241:** 42 - 52, 1988.

7) Kessler, S.: Rapid isolation of antigens from cells with a staphylococcal protein A adsorbent: Parameters of the interaction of antibody - antigen complexes with protein A. **J Immunol 115:** 1617 - 1624, 1975.

8) Krebs, E. G.: The enzymology of control by phosphorylation. **In: The Enzymes, Vol XVII, Control by Phosphorylation, Part A** (eds. E. G. Krebs & Beavo), pp. 3 - 20. Academic Press, Orlando, 1986.

9) Kulkarni, G.., Jagannatha-Rao, G. S., Srinivasan, N. G., Hofer, H. W., Yuan, P. M. & Harris, B. G.: *Ascaris suum* phosphofructokinase: Phosphorylation by protein kinase and sequence of the phosphopeptide. **J Biol Chem 262:** 32 - 34, 1987.

10) Laemmli. U. K.: Cleavage of the structural proteins during the assembly of the head of bacteriophage T4. **Nature 277:** 680 - 685, 1970.

11) O'Farrell, P. H.: High resolution two-dimensional electrophoresis of proteins. **J Biol Chem 250:** 4007 - 4021, 1975.

12) Read, S. M. & Northcote, D. H.: Minimization of the variation in the response to different proteins of the Coomassie Blue G dye - binding assay for protein. **Anal Biochem 116:** 53 - 64, 1981.

13) Srinivasan, N. G.. Wariso, B. A., Kulkarni, G., Jagannatha-Rao, G. S. & Harris, B. G.: Phosphofructokinase from *Dirofilaria immitis*: Stimulation by phosphorylation with cyclic AMP-dependent protein kinase. **J Biol Chem 263:** 34382 - 3485, 1988.

14) Taylor, D. M., Goddard, J. M. & MacMahon, J. E.: Surface Components of *Onchocerca volvulus*. **Mol Biochem Parasitol 18:** 283 - 300, 1986.

15) Titanji, V. P. K., Mbacham, W. F. & Leke, R. G. F.: Lectin - binding properties of *Onchocerca volvulus* developmental stages. **Ann Univ Sc Santé 4:** 396 - 401, 1987.

16) Titanji, V. P. K., Muluh, J. P. & Tchoupé, J. - R.: Studies on glucose - 6- phosphate dehydrogenase from the human parasite, *Onchocerca volvulus*. **Parasitol Res 74:** 380 - 382, 1988.

17) Titanji, V. P. K. & Zetterqvist, Ö.: Detection of protein kinase substrates in extracts of *Onchocerca volvulus*. **Acta Trop 43:** 343 - 348, 1986.

18) Walter, R. D. & Albiez, E. J.: Inhibition of NADP - linked malic enzyme from *Onchocerca volvulus* and *Dirofilaria immitis* by suramin. **Mol Biochem Parasitol 4:** 53 - 60, 1984.

19) Walter, R. D. & Schulz - Key, H.: Interaction of suramin with protein kinase 1 from *Onchocerca volvulus*. In: **The Host Invader Interplay** (ed. H. Van den Bossche), pp. 709 - 712. Elsevier/North Holland Biomedical Press, Amsterdam, 1980.

20) Walter, R. D. & Schulz - Key, H.: *Onchocerca volvulus*: Effect of suramin on lactate

dehydrogenase and malate dehydrogenase. Tropenmed Parasitol 31: 55 - 58, 1984.

21) **World Health Organization:** Announcement. **Biochem Pharmacol 33:** 513 - 514, 1984.

22) **World Health Organization:** In: Report of the Steering Committee of the Scientific Working Group on Filariasis. TDR/FIL/SC - SWG (83 - 88)/88.3, 1988.

23) **Zetterqvist, Ö., Ragnarsson, U. & Engström, L.:** Substrate specificity of cyclic AMP - dependent protein kinase. In: **Peptides and Protein Phosphorylation** (ed. B. E. Kemp), pp. 171 - 187. CRC Press Inc. Boca Raton, 1990.

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