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Biochemical Study of Certain Enzymes and Metabolites of the Carbohydrate Metabolism in the Skeletal Muscle of the Dengue Virus-Infected Mice

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SUMMARY

Changes in enzymes and metabolites of the carbohydrate metabolism in skeletal muscles were studied in mice after intracerebral inoculation of dengue type 2 virus. It was noted that lactic dehydrogenase, aldolase, phosphoglucoisomerase, phosphoglucomutase, GO-T and GP-T activity were enhanced initially by two- to three-fold, reaching a peak on day 5. As the illness appeared in mice, all the enzyme activities were lowered and were about three times less in the paralytic stage on the 8th day as compared to controls. Fructose-1,6-diphosphatase activity was increased on the 4th and 5th days but decreased later. Acid phosphatase increased abruptly from the 6th day while alkaline phosphatase activity was irregular. Creatine increased on the 4th and 5th days but diminished later. Glycogen decreased from the beginning and was lowest on the 5th day, but the levels increased later and were maximum in paralysed muscles. On the other hand, lactic acid began accumulating in the muscles and was maximum on the 5th day, then declined. Dengue virus was detected in the muscles from the 2nd day but higher titres were seen from the 6th day. Changes similar to the preparalytic stage of mice may occur in human beings, causing myalgia.

INTRODUCTION

One of the main features of dengue fever is myalgia, involving mainly the muscles of the back and limbs. This results in extreme muscular weakness and prolonged convalescence (Halstead, 1966; Chaturvedi *et al.* 1970). Therefore, the disease has been described as 'break-bone' disease. The cause of myalgia in dengue virus infection is not known. Myalgia can be produced by damage to the muscle fibres but no significant damage of skeletal muscles was seen histologically in dengue virus infected mice (U. C. Chaturvedi *et al.* unpublished data). Other causes may be anoxia or disturbance in metabolism (Keele & Neil, 1966). A study of the enzymes of the skeletal muscles has been made in mice infected with Coxsackie A virus (Albrecht & Sauthoff, 1954; Albrecht & Gadeke, 1956; Green, 1956). It should be mentioned that Coxsackie A virus causes frank myositis which is not true of dengue virus. In our previous studies we have noted that different types of injurious agents produce disturbances in the metabolism of tissue culture cells and alterations in the enzymes (Chaturvedi *et al.* 1972; Bahuguna & Chaturvedi, 1974; Chaturvedi *et al.* 1975). It was

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therefore considered worthwhile to investigate changes in different enzymes of the skeletal muscles of dengue virus-infected mice in order to elucidate their possible role in myalgia. Some of the enzymes and metabolites concerned in carbohydrate metabolism were chosen for the present study.

METHODS

Mice. Adult albino Swiss mice weighing about 20 to 25 g were used. Mice which died during the course of the experiment were rejected.

Virus inoculated group. Dengue virus type 2 (DV) strain 23085 was obtained from the Virus Research Centre, Poona, and was passed into brain of adult mice. DV suspension prepared from the infected mouse brain was inoculated intracerebrally (i.c.) into adult mice at doses of about 1000 LD_{50}. Mice were sacrificed in groups of 10 animals at 24 h intervals from 0 h up to the 8th day, when the limbs of the mice were completely paralysed.

At each time Hanks' BSS-perfused thigh muscles from two of the mice were collected for titration of virus. A tenfold serial dilution of each specimen was inoculated i.c. into groups of adult mice and the titre was calculated by the method of Reed & Muench (1938) after observing them for 21 days.

Control groups. As controls, animals of group A were given the same amount of u.v.-inactivated dengue virus (u.v. DV) while animals of group B were given normal mouse brain suspension (NMB) intracerebrally. The animals of control groups A and B were sacrificed in batches of five animals at time 0 h and 1, 3, 5 and 7 days after inoculation. Loss of infectivity of the dengue virus in the u.v.-inactivated preparation was checked by intracerebral inoculation into mice. Control group C consisted of normal healthy mice which were sacrificed in batches of 10 animals at 24 h intervals from 0 h to 8 days.

Preparation of muscle homogenate. Mice were given light ether anaesthesia. Muscles of both thighs were excised promptly, stripped of fat and connective tissue and placed in ice-cold 0·25 M-sucrose solution. They were then chopped and homogenized in an MSE homogenizer using sucrose solution and keeping the cup of the homogenizer dipped in an ice bath. The homogenate was centrifuged at 5000 rev/min at 40 °C and the supernatant was collected for enzyme assays. The time between homogenization and enzyme assay was kept the same for all the assays with a given method. Supernatants were stored at −20 °C in sterile vials up to the time of assay and the enzymes were assayed within 72 h.

Estimation of enzymes. Lactate dehydrogenase (LDH; EC 1.1.1.27) activity was measured by the colorimetric method of King (1965) which is based on the formation of pyruvate dinitrophenyl hydrazone. Aldolase (ALD; EC 4.1.2.7) activity was estimated by the method of Sibley & Lehninger (1949) as modified by Beck (1955). Phosphoglucose isomerase (PGI; EC 5.3.1.9) enzyme activity was measured by the colorimetric method of Horrocks *et al.* (1963) in which the enzyme was allowed to react over freshly prepared disodium glucose-6-phosphate at pH 7·8 and the liberated fructose was estimated with resorcinol-thiourea reagent. Phosphoglucomutase (PGM; EC 2.7.5.1) activity was determined by the colorimetric method of Ivie (1964). Fructose-1,6-diphosphatase (FDPase; EC 4.1.2.13) activity was estimated by allowing the muscle homogenate to act on fructose-1,6-diphosphate disodium salt (pH 7·2, 0·05 M in 0·05 M-borate buffer; pH 9·5, in the presence of magnesium sulphate, 0·05 M). Protein was precipitated with 10 % cold trichloroacetic acid (TCA) solution. Liberated phosphorus was determined by the method of Fiske & Subbarow (1925). Creatine was determined by the method of Ennor & Rosenberg (1952), as described by Oser (1976). Acid and alkaline phosphatase activity was determined by the method of King (1965) using *p*-nitrophenyl phosphate as substrate. The glutamyl oxalo-

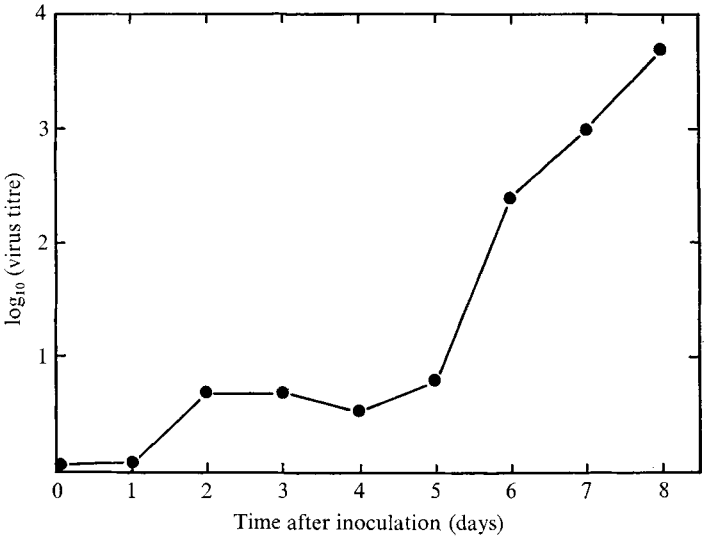

Fig. 1. Virus titres in the skeletal muscle of infected mice on different days after i.c. inoculation. Each point represents the average for two mice.

acetate transaminase (GO-T) and glutamyl pyruvate transaminase (GP-T) activities were determined by the method of Reitman & Frankel (1957), measuring the amount of oxaloacetate formed from aspartic acid or pyruvate from alanine at pH 7·6. Muscle glycogen was extracted by boiling in 30 % KOH for 15 min. The glycogen was precipitated by adding saturated sodium sulphate and 95 % alcohol. The precipitated glycogen was determined by the method of Carroll *et al.* (1956) using anthrone reagent. Lactic acid was determined by the method of Barker & Summerson (1941), after precipitation of proteins.

The enzyme substrates were purchased from Sigma. All the chemicals used were of A.R. grade and de-mineralized water was used throughout the study. The details of enzyme assay have been described earlier (Bahuguna & Chaturvedi, 1974; Chaturvedi *et al.* 1975; Chaturvedi & Bahuguna, 1976).

All the enzyme activities have been expressed in terms of the amount of protein and the amount of glycogen and lactic acid as per g wet weight of the muscle. The concentration of protein in the muscle homogenate was determined by the method of Lowry *et al.* (1951).

RESULTS

Following intracerebral inoculation of dengue virus the mice showed no sign of illness up to the 5th day. On the 6th day there was ruffling of the hair. On the 7th day the mice were sick and by the 8th day most of them were severely ill and the limbs were paralysed. Average virus titres in muscles of two mice at each period after dengue virus inoculation are presented in Fig. 1. The titres increased markedly from the 6th day and on the 8th day it was $10^{3 \cdot 7}$.

Levels of the different enzymes and metabolites in the skeletal muscles of 10 each of the DV infected and normal mice (group C) and 5 each of the u.v.DV and NMB inoculated control groups at each period were pooled and the mean values with ± standard deviation have been presented. The values in u.v.DV and NMB treated mice were similar to those of normal healthy untreated control mice. In DV infected mice, LDH (Fig. 2), aldolase (Fig. 3), PGM (Fig. 4), PGI (Fig. 5), GO-T and GP-T (Table 1) showed increases in the

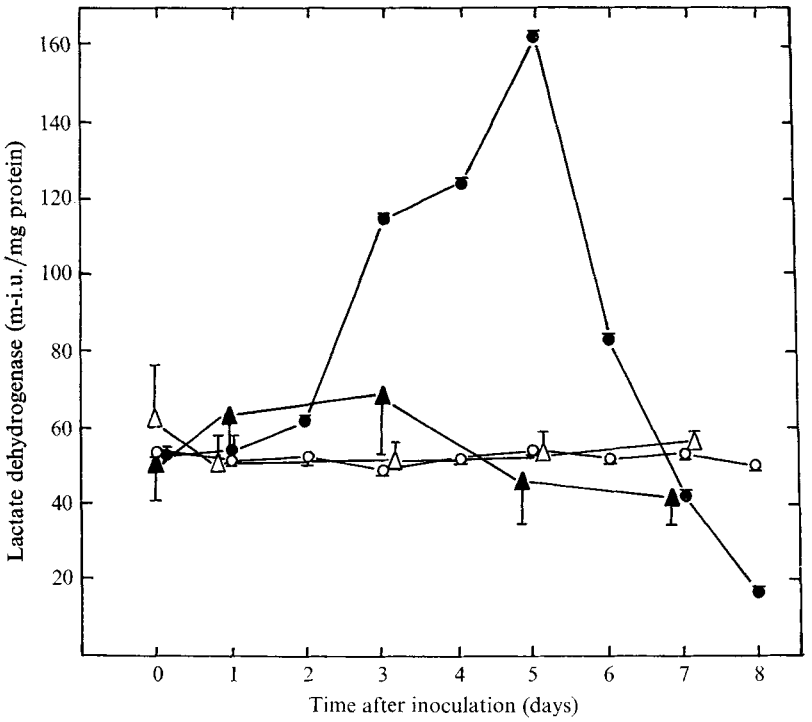

Fig. 2. Lactate dehydrogenase enzyme activity in the skeletal muscle of the DV (●—●), u.v. DV (▲—▲) and NMB (△—△) inoculated mice and normal untreated mice (○—○) on different days after i.c. inoculation. Each point represents the mean value for 10 mice of DV treated or untreated groups and 5 mice each of u.v. DV and NMB treated groups. Bars indicate standard deviation from the mean.

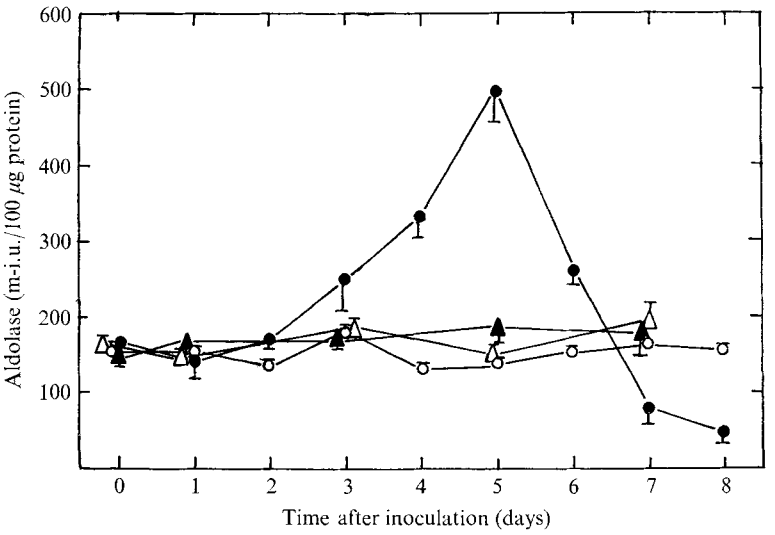

Fig. 3. Aldolase enzyme activity in the skeletal muscle of mice on different days. Symbols are the same as in legend to Fig. 2.

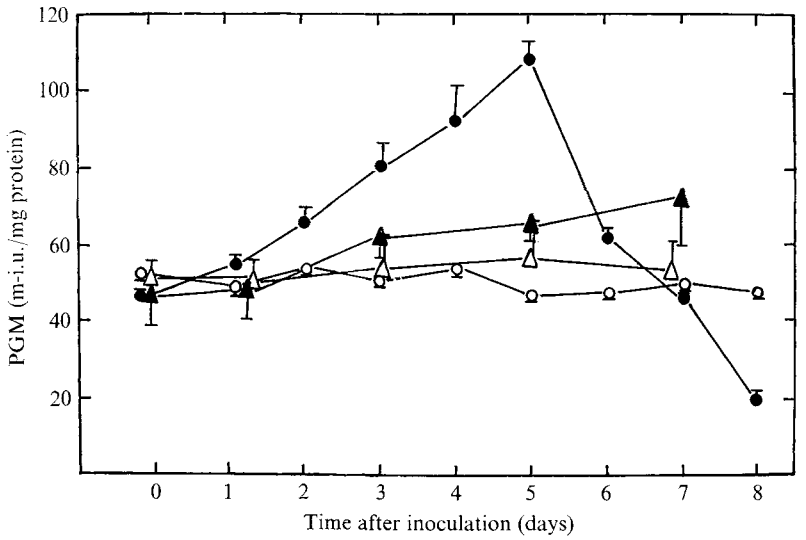

Fig. 4. Phosphoglucomutase enzyme activity in the skeletal muscle of mice on different days. Symbols are as in legend to Fig. 2.

Table 1. *Activity of glutamyl oxaloacetate transaminase and glutamyl pyruvate transaminase in the skeletal muscles of dengue virus-infected mice**

Days after inoculation	GO-T		GP-T	
	Infected mice	Control mice Group B	Infected mice	Control mice Group B
1	5·42±0·08†	5·34±0·04†	5·34±0·12†	5·18±0·16†
2	7·50±0·12	5·50±0·06	6·48±0·10	5·09±0·19
3	11·80±0·21	5·50±0·03	8·00±0·22	5·01±0·18
4	13·6±0·19	5·50±0·07	9·70±0·32	5·18±0·22
5	19·30±0·16	5·82±0·03	15·70±0·46	5·18±0·12
6	8·09±0·09	5·66±0·08	7·76±0·32	5·34±0·17
7	5·50±0·11	5·66±0·02	7·50±0·26	4·70±0·22
8	3·47±0·21	5·50±0·08	5·74±0·32	5·34±0·13

* Activity of GO-T and GP-T was assayed in the muscle homogenate as described in Methods. The control group B included NMB treated mice.

† Mean values in milli-international units (m-i.u.) per 1 mg proteins of the muscle ±s.d.

earlier stages and subsequent significant decreases ($P < 0{\cdot}001$). Unlike these enzymes, FDPase (Fig. 6), which is involved exclusively in the reverse direction, especially gluconeogenesis, is unaffected till the 3rd day, then increased on the 4th and 5th days and again decreased significantly in paralysed muscles ($P < 0{\cdot}001$). The acid phosphatase activity increased abruptly from the 6th day ($P < 0{\cdot}001$) while the pattern of alkaline phosphatase activity was irregular (Table 2). The creatine contents of the muscles increased on the 4th and 5th days and then diminished significantly on the 8th day (Table 3). Glycogen content on the other hand was minimal at 5 days, decreasing steadily from the beginning of infection (Fig. 7). However, the glycogen increased from the 6th day onwards. As expected, lactic acid showed more or less reciprocal behaviour compared to glycogen (Fig. 8). The initial increase in lactic acid up to the 5th day was parallel to that of the glycolytic enzymes and later it also decreased.

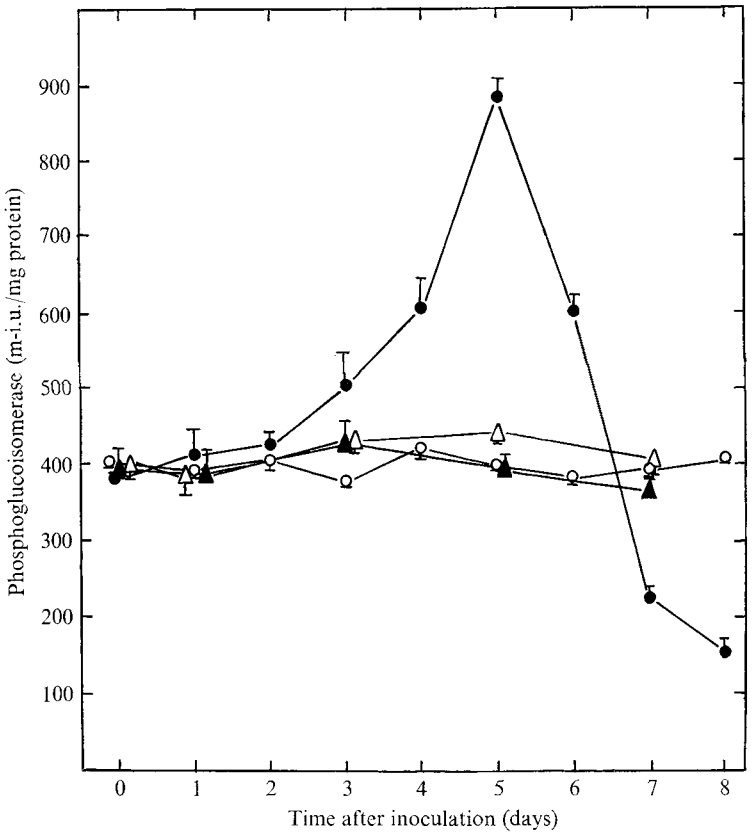

Fig. 5. Phosphoglucoisomerase enzyme activity in the skeletal muscle of mice on different days. Symbols are as in legend to Fig. 2.

DISCUSSION

Our data show that infection of mice with dengue virus has two stages biochemically. The initial phase, lasting up to 5 days, is of an increased glycolysis and glycogenolysis, probably caused by metabolic stress due to virus replication; and the later phase could be a metabolic degeneration due to cell injury.

The increased glycogen content in the later phase may be due to decreased glycogenolysis and to increased glycogenesis, utilizing blood glucose and other carbohydrate sources, through kinase and the UDPG system. Since PGM is always present in a high proportion and is not a regulatory step, its decrease is not likely to affect the synthetic pathway. Excessive glycogen formation takes place in spite of decreased fructose-1,6-diphosphatase, so that gluconeogenesis is not likely to be enhanced. This is apparent also from the decreased transaminase activity during the terminal period when glycogen is highest, so that channelling of glycogenic amino acids towards carbohydrate metabolism is not altered. The increase in transaminases at 5 days could be indicative of the formation of various amino acids for new protein synthesis and channelling of glutamate and aspartate along with glycine for purine and pyrimidine synthesis. Regulatory effects of nucleotides on FDPase and the diffusion of lactic acid due to altered permeability are the other factors likely to be operative.

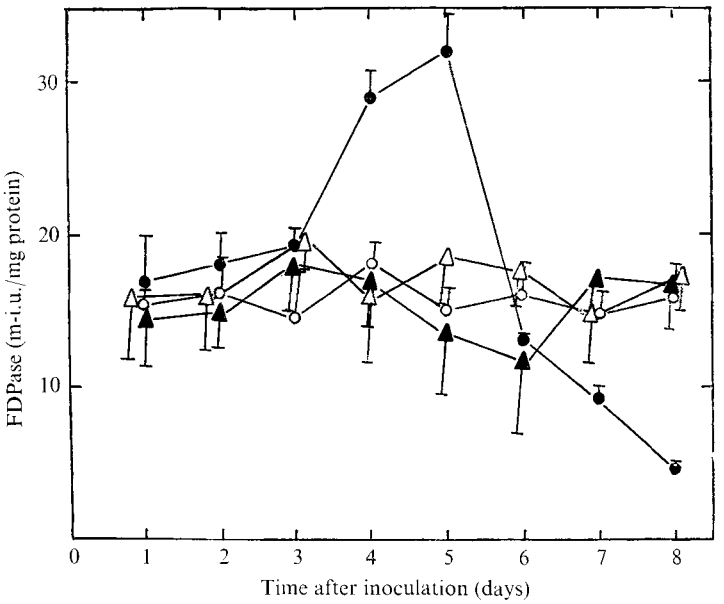

Fig. 6. Fructose-1,6-diphosphatase enzyme activity in the skeletal muscle of mice on different days. Symbols are as in legend to Fig. 2.

Table 2. *Acid and alkaline phosphatase activity in the skeletal muscles of dengue virus-infected mice**

Days after inoculation	Acid phosphatase		Alkaline phosphatase	
	Infected mice	Control mice Group B	Infected mice	Control mice Group B
1	2·8±0·02†	2·40±0·03†	3·83±0·03†	3·54±0·025†
2	2·4±0·01	2·71±0·02	3·4±0·25	3·54±0·03
3	2·36±0·05	2·46±0·03	2·55±0·04	3·4±0·06
4	1·83±0·01	2·34±0·02	4·6±0·06	2·97±0·10
5	2·09±0·02	2·43±0·03	4·18±0·02	3·1±0·12
6	4·6±0·05	2·40±0·02	3·4±0·01	3·68±0·10
7	3·86±0·07	2·83±0·04	2·76±0·42	3·54±0·08
8	4·28±0·05	2·50±0·02	4·46±0·34	3·68±0·06

* Activity of acid and alkaline phosphatase in the muscle homogenate was assayed as described in Methods. The control Group B included NMB treated mice

† Mean values in m-i.u. per 1 mg of the protein contents of the muscles ±s.d.

During their study on virus infected cells, Allison & Sandelin (1963) showed that lysosomal enzymes may cause breakdown of host cell polynucleotides resulting in a markedly increased pool of acid soluble nucleotides in infected cells. They also observed a concomitant enhancement of glycolysis in virus infected cells which favour our observations. Green (1956) has also observed reduced glycolytic activity in paralysed muscles of Coxsackie A virus infected mice. Stimulation of glucose catabolism in Semliki Forest virus-infected cells (Cassells & Burke, 1973) supports our findings in dengue virus-infected mice. Our findings of decreased creatine in later stages resemble the observations of Gifford & Dalldorf (1949) with Coxsackie virus.

Table 3. *Creatine contents in the skeletal muscles of dengue virus-infected mice.**

Days after inoculation	Infected mice	Control mice		
		Group A	Group B	Group C
1	0·76±0·21†	0·92±0·31†	0·86±0·23†	0·83±0·22†
2	0·82±0·16	0·88±0·23	0·92±0·42	0·76±0·18
3	0·69±0·10	0·86±0·14	0·76±0·21	0·72±0·14
4	1·14±0·32	0·62±0·12	0·72±0·16	0·81±0·21
5	1·99±0·66	0·76±0·20	0·80±0·13	0·76±0·24
6	0·41±0·10	0·71±0·32	0·56±0·13	0·68±0·36
7	0·22±0·04	0·69±0·32	0·82±0·32	0·75±0·32
8	0·17±0·07	0·72±0·16	0·70±0·20	0·82±0·43

* Thigh muscles of infected mice were processed and the creatine assayed in the muscle homogenate as described in Methods. Control group A was given ultraviolet inactivated dengue virus i.c., control group B was given normal mouse brain and the control group C included untreated mice.

† Mean values in mg per g of fresh weight of the tissue ± s.d.

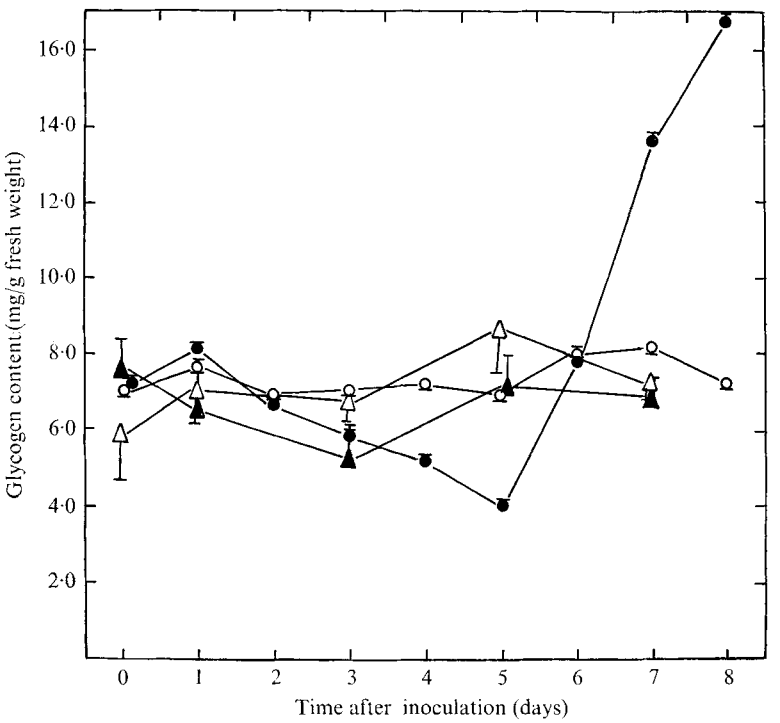

Fig. 7. Glycogen content in the skeletal muscle of mice on different days. Symbols are as in legend to Fig. 2.

The increase in acid phosphatase in the later stages indicates involvement of lysosome in the degenerative processes. This is supported by our electron microscopic studies on DV infected muscles (personal unpublished data). The irregular pattern of alkaline phosphatase cannot be explained at present, but it could be the effect of the altered level of phosphorylated intermediates and the need for regulating the availability of inorganic phosphorus required for glycogenolysis and phosphoglyceraldehyde dehydrogenase.

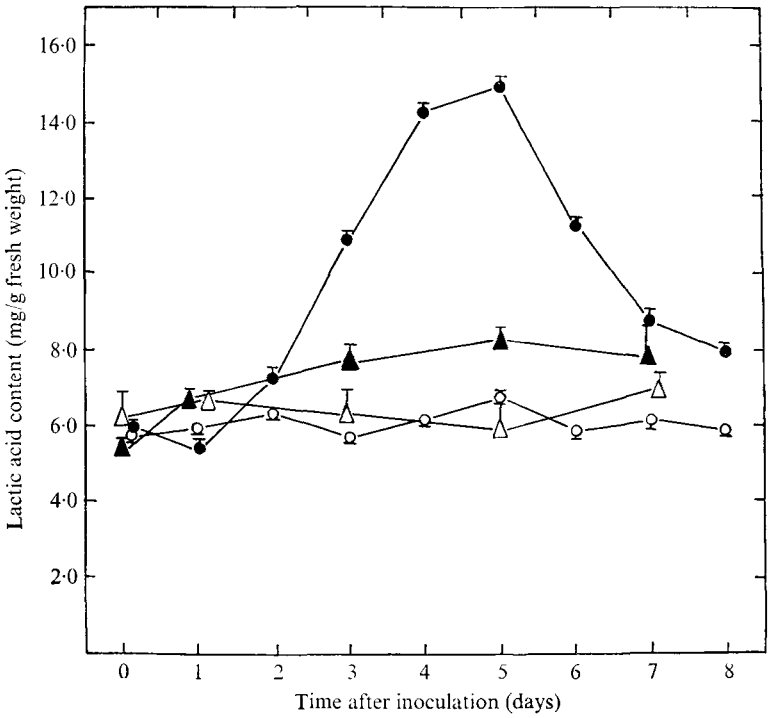

Fig. 8. Lactic acid content in the skeletal muscle of mice on different days. Symbols are as in legend to Fig. 2.

Thus, increases in glycolytic enzymes and the end product of their metabolism (lactic acid) reach a peak on the 5th day, before the onset of apparent illness in mice. Damage to subcellular organelles of tissue culture with consequent changes in their enzymes occur long before morphological expression of injury by viruses, u.v. irradiation or bacterial toxins (Chaturvedi *et al.* 1972, 1975; Bahuguna & Chaturvedi, 1974). Localization of virus occurs in the brain, liver, heart and spleen of DV infected mice (Chaturvedi *et al.* 1974, 1977, 1978), and in the skeletal muscles as shown in the present study and also by Marchette *et al.* (1972) in the monkeys. Alterations in the enzymes and metabolites observed in the present study, therefore, besides being due to cellular stimulation by the virus, may also be due to damaged subcellular organelles associated with the replication of DV. This is supported by our observations of (i) subcellular damage as seen electron microscopically and (ii) similar enzyme changes in liver, etc. of DV infected mice (D. K. Agrawal *et al.* unpublished data).

In human beings dengue virus causes fever and myalgia, etc. but no paralysis. It is likely that enzyme changes similar to the pre-paralytic stage of mice occurs in humans, also causing severe myalgia and weakness.

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