

## PRELIMINARY RESULTS ON ELECTROPHORETIC AND IMMUNOELECTROPHORETIC FRACTIONATION OF BOVINE MUSCLE EXTRACT

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**OPSOMMING:** VOORLOPIGE RESULTATE VAN ELEKTROFORETIESE EN IMMUNOELEKTROFORETIESE SKEIDING VAN BEES-SPIEREKSTRAK.

Besonderhede van tegnieke van styselgel-, sellulose asetaat- en immunoelektroforese is gegee, wat 'n skeiding van spierekstrak op so 'n manier moontlik maak, dat variasies tussen individuele monsters kwalitatief opgespoor kan word. Die reproduseerbaarheid van resultate by styselgel- en sellulose-asetaat elektroforese was besonder goed terwyl klein variasies in die tegniek sekere analises by immunoelektroforese bemoeilik het. Maar ook hier kom later 'n goeie reproduseerbaarheid van resultate teweeggebring word. Die volgende stap in hierdie studie is die bepaling van die oorerwing van die fraksies wat deur die drie tegnieke geskei kon word, en die ondersoek van spierproteïen veranderinge in kalwers met spastiese paretse.

### SUMMARY

Details of techniques are presented of starch gel-, cellulose acetate - and immunoelectrophoresis which can be used to fractionate bovine muscle extract in such a way that the variation between individual samples can be detected qualitatively. The repeatability of results in starch gel- and cellulose acetate electrophoresis was very good whereas small variations in the technique of the immunoelectrophoresis hindered correct analysis. Later, however, clear repeatability of results could also be obtained here. The following step in this study will be the determination of the hereditary background of these fractions separated by the three techniques, and the investigation of muscle protein changes in calves with spastic paresis.

The first electrophoretic studies on the proteins of muscle tissue were carried out by Bosch (1951), who found that differences existed between muscles from different animals as well as between muscles from the same animal. Hughes (1959, 1960) performed similar studies on human muscle tissue and identified different myoglobin and myoalbumin fractions. He found that quantitative differences between these two fractions were related to muscle atrophy due to denervation. Kronman, Weinberger & Winterbottom (1960) investigated the biochemical and electrophoretic differences between eight different bovine muscles and found that they differed with respect to water soluble proteins, aldolase and ultimate pH. Quantitative differences also existed amongst the various protein fractions of different muscles together with variations in the electrophoretic patterns. Kossmann (1969) found great individual differences in the esterase in his pherograms of proteins of bovine skeletal musculature and was able to establish genetic polymorphism for two esterase systems. From a phylogenetic point of view, Giles (1962) investigated the differences between the sarcoplasmic proteins of beef, mutton and pork, and demonstrated a similar pattern for ruminants in contrast to the pig. Similar work has been done in sea vertebrates, namely by Tsuyki, Roberts & Gadd (1962) on the Pacific salmon and by Hapner, Bradshaw, Hartzell & Gurd (1968) on the seal, porpoise and whale, using electrophoretic procedures. Their findings, like those of Hartshorne and Perry (1962) revealed distinct phylogenetic as well as ontogenetic differences in the sarcoplasm. The object of the present study was to extend the practical applications of biochemical genetic typing to an investigation of muscle proteins from cattle.

### Procedure

Specimens were collected from the following muscles: *M. gastrocnemius*, *M. biceps femoris* and *M. brachiocephalicus*, the first two being those affected by spastic paresis. Spastic paresis is a deleterious trait with a genetic background which is causing increasing concern in certain cattle breeds. The muscle specimens were obtained from 72 normal animals whilst they were still on the hook in the process of being skinned, i.e. pre-rigor muscle samples. Being aware of the relatively quick changes in pH, lactates and energy-rich phosphates small pieces of approximately 20 g were dissected out from the three muscles and treated immediately in the following way: after transporting the samples in phosphate buffered saline to the laboratory - a distance of only 300 meters - the specimens were cut into fine pieces and swung down in an ultra-centrifuge at 100 000 g, according to the technique of Kossmann (1969). This process was also carried out as soon as possible so as to avoid excess denaturation and consequent pH changes (Scopes & Lawrie, 1963). After 30 mins. the samples were removed and the supernatant (approximately 4 ml for every 14 g meat) was decanted and either processed immediately or stored at -15°C for use the following day.

Starch gel electrophoresis was carried out using the procedure of Kossmann but with the following buffers: for the gels, 3,6 g Trishydroxymethylaminomethane and 0,8 g citric acid made up to 1 litre with distilled water, and for the electrodes, 1, 2 g LiOH and 11,8 g Boric acid made up to 1 litre with distilled water. Using 24 g Connaught hydrolysed starch in 186 ml gel buffer, the gels were cast in perspex molds measuring 24 cm x 15,5 cm x 3 mm. A current of 400 volts and 50 mA was applied until the borate boundary had migrated ca. 10 cm, after a period of ca. 3 hours. Care

was exercised in keeping the gels cool during the process as excess heat tended to give illegible results. On completion of the run, each gel was removed from its tray and stained with amidoschwarz 10B, the patterns being read on the underside.

Zone electrophoresis was also done on cellulose acetate membranes so as to obtain clear separation of the myoglobins. In the work of Kossmann (1969) no variation could be found in this fraction. Using a buffer composed of 6,2 g Trishydroxymethylaminomethane, 1,6 g citric acid made up to 1 000 ml with distilled water, and a current of 166V and 4 mA, the membranes were stained with benzidine after a running time of 1<sup>1</sup>/<sub>2</sub> hours.

Immunoelectrophoresis was performed using the micro-technique of Grabar and Burtin (1964), and a continuous barbitone-acetate buffer system, ionicity 0,1; pH 8,6. Electrophoresis was carried out at 250 volts and 25 mA for 2 hours. Precipitation was accomplished with rabbit anti-bovine serum (RAB 20) and a pooled antiserum, the minimum reaction time being 16 hours. Excess antigen took about 48 h to dialyse out sufficiently to give a clear background, presumably due to the high protein and fat content of the extract.

For the production of suitable precipitating antisera, it was decided to alter the technique as applied by Ward-Cox (1971) as follows: meat juice was injected into five rabbits in 5 ml aliquots weekly for two weeks followed by alum-precipitated serum globulin on the third week and whole serum on the fifth week. On the sixth week they were bled, the antibodies having been controlled on immunodiffusion plates. After a further immunoelectrophoretic control, making use of the strongest antisera only, these were pooled to form the final product.

### Discussion of Results

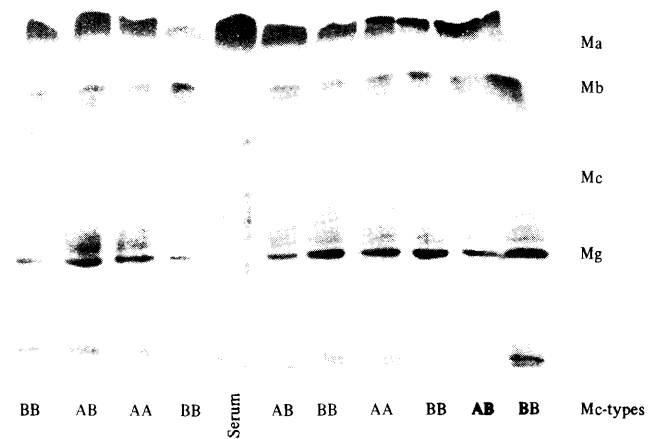
**Zone electrophoresis in starch gel:** From Fig. 1 it is evident that the main fractions along the migration route (the insertion line is indicated on the lower side of the photograph) fall into three clear categories, appearing as the more intensely coloured bands on the electropherogram and marked, in order of increasing migration rate, Mg, Mb and Ma, Mg is clearly the myoglobin fraction of Hughes (1959) due to its peroxidase activity as revealed by staining with benzidine. Using a bovine serum control, it was further ascertained that the fastest band Ma corresponded to the albumin, also in agreement with Hughes. The zone Mb could not be differentiated, but from the immunoelectrophoretic patterns it appears that it is closely related to the serum transferrins. However this was not verified by making use of Fe-binding estimates. It can be seen that this zone migrates, somewhat faster than the serum transferrins, and unlike the transferrins, it shows no variability from one individual to another, using this specific technique. The only variation that was observed occurred in the Mc-zone migrating midway between the Mg-zone and the Mb-zone, but which was still slower than the serum transferrins. This

is also in agreement with Kossmann (1969). However, he found only the two types which could be postulated as being the heterozygote and the faster-moving homozygote, whereas this investigation revealed the second homozygote as well. For the sake of convenience they are termed McAA, McBB and McAB. The actual results obtained by starch gel electrophoresis are presented in Table 1.

**Table 1**

*Classification of investigated muscle samples*

	Percentage of different types		
	McAA	McBB	McAB
<i>M. gastrocnemius</i>	59,1	22,7	18,2
<i>M. biceps femoris</i>	50,0	15,4	34,6
<i>M. brachio-cephalicus</i>	34,5	31,0	34,5



**Fig. 1.** — *Starch gel electropherogram of bovine muscle extract*

The actual importance of these different types has not yet been investigated, and await genetic studies to prove whether another polymorphic system is heritable.

**Zone electrophoresis on cellulose acetate:** Myoglobin typing using this technique revealed two distinct types, viz. a single banded and a double banded formation, which showed a variation (see Fig. 2). One specimen gave a slower moving band which may have been due to denaturation and can thus not be considered at this stage. There appeared to be no qualitative variation between muscles of the same animal but clear quantitative differences were obvious as in Fig. 3, where it can be seen that the *M. biceps femoris* showed more intense myoglobin bands and the *M. brachio-cephalicus* the least intensity.

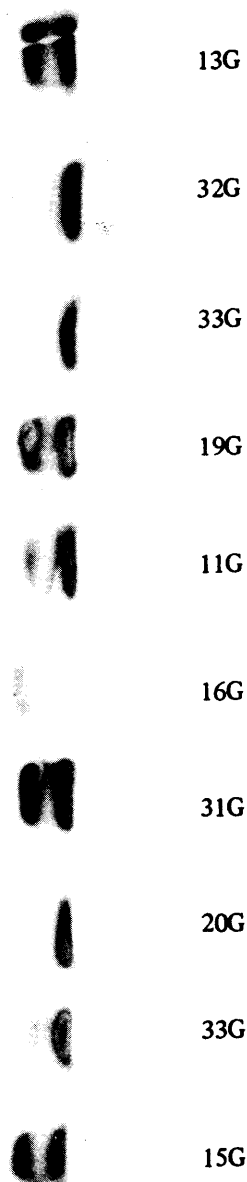


Fig. 2. — Myoglobin types of *M. Gastrocnemius* in different animals

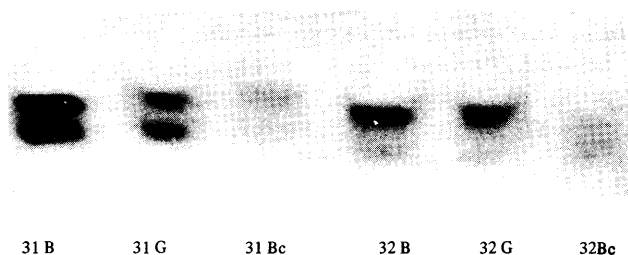


Fig. 3. — Myoglobin types of three different muscles in two animals

The results of the actual typing showed that in 80,6% of all samples of *M. gastrocnemius*, the myoglobin appeared in two bands, while 19,4% showed only one band. In the samples of *M. biceps* 88,9% possessed two bands and only 11,1% a single band, while the myoglobin concentration in the *M. brachio-cephalicus* was too weak to enable clear separation.

**Agar gel immunoelectrophoresis:** A variety of fractions exhibit immune patterns in this technique, as can be seen from Fig. 4 where an attempt has been made to depict all the precipitin arcs that were observed. There were three major arcs, as was also the case in the starch gel patterns where three major zones could be seen. On the basis of their migration properties as compared to the starch gel patterns, the terms Ma, Mg and Mc were also used. The arcs Mg and Mc appear as either single or double formations. The variation of the Mc are coincided with that of its counterpart on starch gel although it was not possible at this stage to distinguish between the homo- and heterozygotes by this technique. The variations of the Mg arc cannot, however, be correlated with any similar phenomenon on starch gel. The small differences that Kossman considered too insignificant to take into account may be explained by this method, as he carried out specific tests on a variety of myoglobin samples.

When compared with the immunoelectrophoretic pattern obtained with whole serum, as in Fig. 5, remarkable similarities occurred. Although the original samples were taken into PBS (phosphate buffered saline) and thoroughly washed in this solution before processing, it was therefore impossible to set a clear differentiation between muscle and vascular protein fractions although there many differences exist. Where serum (b) showed up to thirty-two arcs, the muscle proteins (a) showed only a maximum of ten, including the variations of the Mg and the Mc arcs. Generally speaking, the arcs of serum protein fractions were more extensive than those of meat extract. This was most obvious in the albumin, transferrin (siderophilin) and  $\gamma$ -globulin arcs. Certain major serum protein arcs were completely lacking in meat extract, e.g. the  $\alpha_2$ -macroglobulin (c) which had its origin in the antigen well and extended towards the albumin arc.

In accordance with the general patterns obtained with starch and agar, the major variations occurred within the three main arcs, as illustrated in Fig. 6. There were three combinations in the samples to be found, viz. the Ma arc alone (28B), the Ma and the Mg arcs (19 BC, 31 G, 39 B), and the Ma, Mc and Mg arcs (62G, 38 B). More arcs could actually be seen but these were the clearest ones found in the extracts of the three muscles used. Furthermore, these types were distributed amongst all the muscles but detailed genetic analysis has still to be carried out. It was noticeable that the Ma arc was always present but the Mg and the Mc arcs were absent in many instances. The quantitative differences previously mentioned were very pronounced in the Ma arcs but not in the others. This fact has been observed in all the material investigated and is shown in Fig. 7.

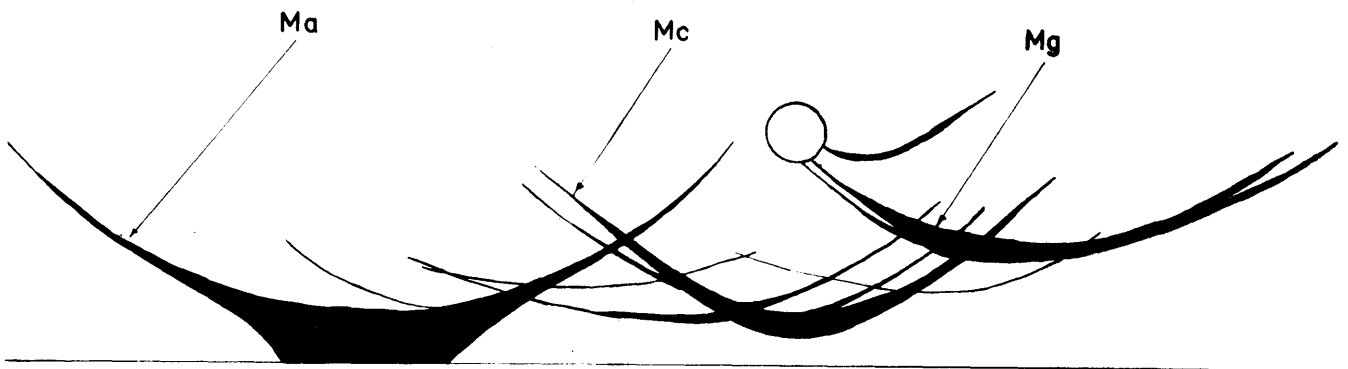


Fig. 4. — Diagram of immunoelectrophoretic fractions of muscle extract

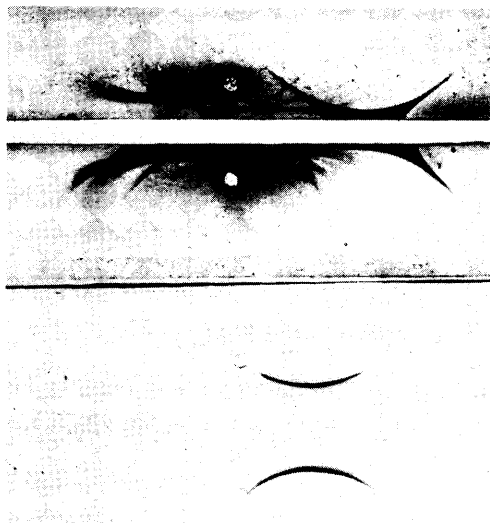


Fig. 5. — Comparison of immunoelectrophoretic patterns of (a) muscle extract, (b) blood serum and (c)  $\alpha_2$ -macroglobulin

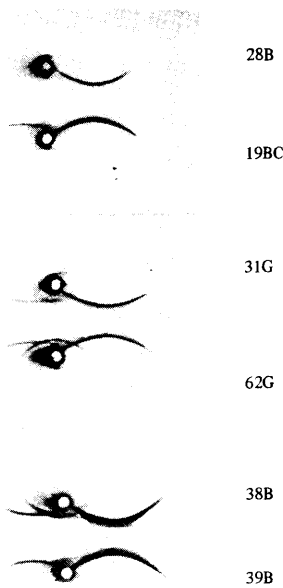


Fig. 6. — Various combinations of three major immunoelectrophoretic fractions of muscle extract after a running time of 1 1/2 hours

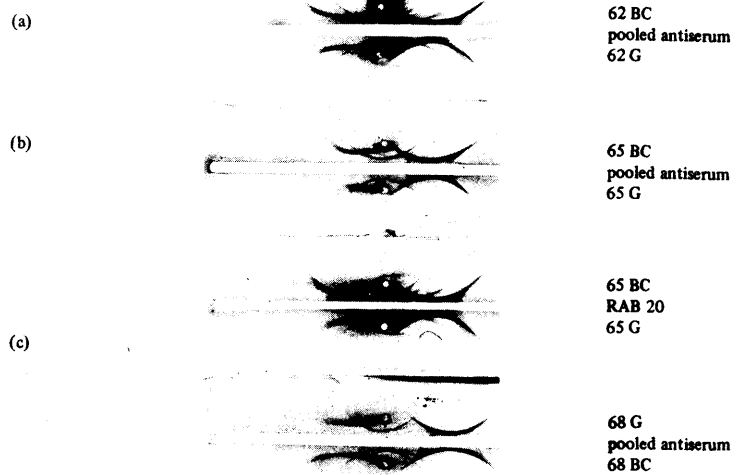


Fig. 7. — Variation of the immunoelectrophoretic fractions in two muscles of the same animal after a running time of 2 hours

The difference in the mobility between the Mg arcs of two muscles from one animal, as in slide 13K of Fig. 7, although not observed in blood serum using the same technical procedure, may be attributed to the same phenomenon as observed by Nansen (1970). Using blood serum he has classified immunoglobulins in terms of electrophoretic mobility as being IgG fast and IgG slow. This nomenclature has not been finally established. However, in spite of the fact that the denaturation of muscle proteins was an uncontrolled factor in this specific type of work, good repeatability of results was obtained in all three techniques. The immediate treatment of the pre-rigor muscle samples apparently helped to overcome the initial difficulties experienced especially in the immunoelectrophoretic technique. Summarising the significance of these findings one could mention the following practical applications:

- a. From anatomical studies performed at Onderstepoort it appears that *M. biceps femoris* and especially *M. gastrocnemius* are involved in the abnormal muscle development in cases of spastic paresis

in calves. This disease expresses itself in an uncontrolled stiffness of one or both hindlegs and is apparently inherited via an autosomal semi-lethal recessive gene. The possibility that the heterozygous carriers of this trait could be established by taking samples from suspected living carriers with a biopsy needle needs to be investigated.

- b. Using the techniques described a further objective determination of some of these qualities of meat which are difficult to measure, could be established, e.g. colour, juiciness and texture. The next step would then be a correlation of the clearly established variants of muscle extracts with these quality characteristics. It must be borne in mind, however that the sarcoplasmic proteins are labile and readily denatured under differing pH and temperature conditions. The next step would then be an investigation of the hereditary background of the established types as mentioned above.
- c. There remains the possibility of using these techniques in the more remote fields of immunogenetics, namely phylogenetic systematics and biosystematics. Until recently, immunogeneticists have restricted their pool of genetic markers to those on the red and white blood cells and to the serum proteins and enzymes. If, for example, the myoglobins, could be included in these systematic classifications breed relationships and species classifications could be performed on an even broader basis.

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