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Chemical Changes in Skeletal Muscle During Development

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Skeletal muscle has been investigated from many angles, and certain aspects of the effect of development on its composition have received a good deal of attention. It is well known, for instance, that during development the percentage of water in skeletal muscle falls (Needham, 1931; Yannet & Darrow, 1938; Hines & Knowlton, 1939; McMeekan, 1940; McCance & Widdowson, 1956*a*) and that this change is accompanied by a fall in the concentrations of the extracellular ions sodium and chloride. At the same time there is an increase in the percentage of protein (Needham, 1931; McCance & Widdowson, 1956*a*) and of the intracellular constituents potassium and phosphorus. These changes show that development is associated with a decrease in the percentage of extracellular fluid and an increase in the 'cell mass'. It is not known if there is any change in the composition of the muscle cell with development, and the present study was undertaken with this objective in view.

Muscle cells cannot be separated for direct analysis, and their composition must be determined indirectly. McCance & Widdowson (1956*a*) have shown that the amount of nitrogen per unit weight of potassium in skeletal muscle increased with development and have interpreted this as possibly indicating a change in the gross composition of the muscle cell. There are, however, two sources of uncertainty about this. First, it has been suggested that more of the potassium is outside the cells in the foetus than in the adult (Widdowson & McCance, 1956) and, secondly, that changes in the proportion of the extracellular proteins collagen, elastin and reticulin may take place during development. Robinson (1952*a*) has shown that such changes do in fact take place in the muscle of the developing chick.

Little information appears to be available about the changes in the proportion of different proteins

in developing mammalian muscle. Hermann & Nicholas (1948) separated a number of protein fractions from the muscle of the rat at various ages, but the method of separation used by these workers did not effect a complete separation of the intracellular from the extracellular proteins. The method devised by Robinson (1952*a*) did effect such a separation, and at the same time enabled the intracellular proteins to be separated into two groups consisting of the sarcoplasmic and fibrillar proteins. This method has now been applied to the muscle of man and the pig at various stages of development in order to ascertain if there is any change in the protein composition of the cell during this process, and at the same time to provide a more reliable basis of reference for other intracellular substances.

EXPERIMENTAL

Materials

Human. Thigh muscle was obtained from four foetuses of approx. 14 weeks' gestation, weighing about 30 g., and from four foetuses of 18–24 weeks' gestation, weighing between 200 and 300 g. The foetal age was calculated from the crown–heel length by means of the formula derived by Scammon & Calkins (1929). The foetuses were all obtained at hysterotomy, pregnancy being terminated for various reasons. Thigh muscle was also obtained from four full-term newborn babies which failed to respond to resuscitation, three babies aged 4–7 months who were suffocated in their cots, one 11-year-old boy and one 16-year-old boy and from four men who were killed in street accidents. The ages of the men were 21, 27, 40 and 43 years.

The foetuses were brought to the Laboratory, generally still in their membranes, and they were dissected within an hour of delivery. Muscle was taken from the seven babies, the two boys and the four adults at autopsy, the bodies being stored in a refrigerator meanwhile.

Pig. The pigs came from the Laboratory farm. The stock was originally Large White × Essex cross, subsequently

mated with pure-bred Large-White boars. Six ages have been studied. There were five litters of foetuses of 46 days' gestation, two litters of foetuses of 90 days' gestation, twelve newborn piglets from six litters, two piglets aged 3 weeks, three aged 4-6 weeks and five pigs aged 8-18 months, which were regarded for the present purposes as adult. Four of the adult pigs were killed at the slaughterhouse and one died under an anaesthetic during a Caesarian section. The newborn and older piglets were killed at the Laboratory with pentobarbitone sodium (Nembutal). The mothers of the foetuses were killed at the slaughterhouse, the uterus was removed and brought to the Laboratory for further dissection. Thigh muscle was removed from all these animals. In the foetuses all the samples from one litter were pooled, but for the newborn and older pigs muscle from individual animals was analysed.

Methods

So far as possible the material was analysed immediately after it was removed from the body. Muscle samples not dealt with at once were stored in sealed containers at -20° . Care was taken when these were thawed out to include in the sample all the fluid that had come out of the tissue during storage.

The muscle was freed from all visible fat and tendon and finely cut with scissors. Duplicate samples of 0.5-2.0 g. were taken for chloride estimation, which was determined as described by McCance & Widdowson (1956*b*). Similar samples were weighed into Universal containers (Baird and Tatlock Ltd.) for total N determinations. These were covered with concentrated H_2SO_4 and left to disintegrate. This took about a week at room temperature, but the process was hastened by heating in an oven at 100° overnight. The mixture was made up to 25 or 50 ml. with water in a graduated flask, and 5 ml. taken for determination of N by the micro-Kjeldahl method. The sample was digested with 2 ml. of conc. H_2SO_4 , with copper selenite as catalyst. The samples for water and ash were weighed into silica crucibles and dried in an oven at 100° to constant weight. They were then ashed in a muffle furnace at 450° and the ash was extracted with HCl as described by McCance, Widdowson & Shackleton (1936). Sodium was determined in the acid extract of the ash with a Beckman flame photometer, after preliminary treatment as described by Widdowson & Southgate (1959), and K, Ca and Mg as described by McCance & Shipp (1933). Phosphorus was also determined on the acid extract of the ash by the method described by King (1932), after heating at 100° for 1 hr. with concentrated H_2SO_4 (1 drop/ml.) to convert all pyrophosphate into orthophosphate. Results obtained by this method agreed well with those obtained after a wet digestion.

The methods used for separating the various protein fractions are being described fully by Dickerson (1960). The percentage of hydroxyproline estimated by Neuman & Logan's (1950) procedure was converted into percentage of collagen on the assumption that human and pig collagen contained 14.1 and 13.5% of hydroxyproline respectively (Eastoe, 1955) at all ages. Collagen was reckoned to contain 18% of N.

Description of the nitrogenous constituents

By the use of appropriate extractants the N of skeletal muscle has been divided into a number of fractions. The 'non-protein N' has been assumed to represent the N in the

tissue which is present in true solution, and this has been separated from the total protein by its solubility in trichloroacetic acid. Beside urea, creatine and purines, this fraction also includes amino acids and some polypeptides, which may be considered as protein in the dietary sense (McCance & Shipp, 1933), but which are not precipitated by this reagent. The nitrogenous compounds not soluble in trichloroacetic acid will be referred to as 'total protein', though it is appreciated that it is really protein plus nucleic acids. The 'total protein' has been divided into three fractions which, though complex, may be considered to have an anatomical significance. The fraction which is designated 'sarcolemmal' is thought to consist of the proteins of the sarcolemma and contains among other things the enzymes of the glycolysis cycle and most of the ribonucleoprotein (Robinson, 1952*a, b*). The 'fibrillar' fraction contains the true fibrillar proteins and their denaturation products and also mucopolysaccharides and deoxyribonucleoprotein (Robinson, 1952*a, b*). The sarcolemmal and fibrillar proteins together constitute the intracellular protein. The extracellular proteins, collagen, elastin and reticulin, constitute a further protein fraction, and one of these, collagen, has been estimated independently.

RESULTS

Nitrogenous constituents

Tables 1 and 2 show the effect of development on the concentration of non-protein and protein N in human and pig muscle. The protein N is further divided into its intra- and extra-cellular components, and the intracellular component is again subdivided into the sarcolemmal and fibrillar fractions. The number of muscle samples studied at each age is shown in the tables.

The concentration of non-protein N increased in the early stages of development in both species, but by the age of 4-7 months in man and 4-6 weeks in the pig it had reached its adult level.

In both species the concentration of total protein N increased with development up to a value which was similar (28 g./kg.) for the adult muscle of man and the pig. The muscle of the newborn baby, however, had a higher concentration of protein N than that of the newborn pig and in this respect the human baby is chemically more advanced than the piglet at birth.

In spite of the increase in concentration of protein N from 9.7 to 18.5 g./kg. during foetal life in man, the concentration of sarcolemmal protein changed little, and in the pig there was actually a decrease in the concentration of this fraction. During the post-natal development of both species the concentration of sarcolemmal protein increased, the value for the adult pig being a little higher than the value for adult man.

The concentration of fibrillar protein increased with development and was higher than that of the sarcolemmal protein at all stages in both species except in the muscle of the 46-day-old pig foetus.

This last-named muscle is less mature than that of the youngest human foetus as judged by the lower concentration of total protein. In the adult of each species the concentration of fibrillar protein was the same. Complex as the sarcoplasmic and fibrillar fractions undoubtedly are, it is evident that they change independently of each other during the foetal period, indicating that during development changes in the composition of the muscle cell take place, at any rate in its protein components.

The highest value for extracellular-protein N was found in the muscle of the human infants and piglets during the suckling period. The concentration of extracellular-protein N in adult muscle was much lower and was similar in the two species. The

collagen N changed in parallel with the extracellular N but, since it forms only a part of the latter, it was lower than the total extracellular N except at two ages in the pig. The explanation of these discrepancies in the pig may lie in small variations in the hydroxyproline content of collagen which have been shown to take place with age in some species (Hall & Reed, 1957).

The distribution of N in the various protein and non-protein fractions has been calculated for each individual sample and expressed as a percentage of the total N. The percentages have been averaged and the results are shown in Tables 3 and 4. These results, and those given in Tables 1 and 2, are analytical values, and for that reason their sum does not necessarily agree exactly with the value

Table 1. *Concentration of nitrogen in the various fractions in human muscle*

Values are expressed as g./kg. of fresh muscle; average and range are given.

	Foetus		Baby		Adult
	14 weeks	20-22 weeks	Newborn	4-7 months	
No. of samples	1	2	2	3	3
Non-protein N	1.2	1.7 (1.3, 2.0)	2.4 (2.3, 2.5)	3.2 (2.8-3.7)	3.0 (2.8-3.2)
Protein N	9.7	13.5 (13.1, 13.9)	18.5 (18.5, 18.5)	25.8 (25.3-26.1)	27.5 (26.7-28.4)
Sarcoplasmic-protein N	3.6	3.7 (3.6, 3.8)	3.9 (3.7, 4.1)	5.0* (4.6, 5.4)	6.7 (6.1-7.5)
Fibrillar-protein N	5.7	8.7 (8.5, 8.8)	10.9 (10.4, 11.3)	17.0* (15.4, 18.5)	19.9 (19.7-20.3)
Alkali-insoluble = extracellular-protein N	0.6	1.8 (1.6, 2.0)	3.8 (3.1, 4.4)	4.6* (4.2, 5.0)	1.4 (0.8-1.7)
Collagen N	—	1.3 (1.0, 1.6)	2.8 (2.8, 2.8)	2.9* (2.6, 3.1)	1.0 (0.6-1.3)

* Two results only.

Table 2. *Concentration of nitrogen in the various fractions in pig muscle*

Values are expressed as g./kg. of fresh muscle; average and range are given.

	Foetus		Piglet			Adult
	46 days	90 days	Newborn	3 weeks	4-6 weeks	
No. of samples	2*	2*	3	2	3	2
Non-protein N	1.1 (1.0, 1.1)	2.0 (1.9, 2.0)	2.6 (2.3-2.8)	3.5 (3.4, 3.6)	4.1 (3.5-4.7)	4.3 (3.7, 4.8)
Protein N	7.2 (7.0, 7.4)	9.4 (8.8, 10.0)	11.0 (10.0-11.6)	25.6 (25.4, 25.8)	23.2 (23.0-23.5)	28.4 (27.1, 29.7)
Sarcoplasmic-protein N	4.0 (4.0, 4.0)	2.4 (2.0, 2.7)	2.2 (1.9-2.4)	5.4 (5.3, 5.5)	5.2 (4.6-5.5)	8.0 (7.7, 8.3)
Fibrillar-protein N	2.5 (2.0, 2.9)	6.1 (6.0, 6.1)	6.5 (5.9-7.4)	16.4 (15.1, 17.7)	16.1 (15.2-16.8)	19.8 (19.1, 20.4)
Alkali-insoluble = extracellular-protein N	0.4 (0.4, 0.4)	1.0 (1.0, 1.0)	2.3 (1.8-3.0)	3.2 (2.0, 4.4)	2.0 (1.8-2.4)	1.1 (0.9, 1.2)
Collagen N	0.4 (0.4, 0.4)	1.2 (1.1, 1.2)	1.3 (1.1-1.5)	3.0 (2.0, 4.0)	1.9 (all 1.9)	1.2 (0.8, 1.5)

* Pooled samples of muscle from several foetuses.

Table 3. *Distribution of the nitrogen in human muscle*

Results of Table 1 are here expressed as a percentage of the total N.

	Foetus		Baby		Adult
	14 weeks	20-22 weeks	Newborn	4-7 months	
Non-protein N	11.0	11.0	11.5	11.0	9.7
Sarcoplasmic-protein N	33.5	24.3	18.6	17.0	22.0
Fibrillar-protein N	52.5	57.1	52.1	57.6	65.6
Alkali-insoluble = extracellular-protein N	5.5	11.8	18.2	15.6	4.6
Collagen N	—	8.3	13.7	9.6	3.3

Table 4. *Distribution of the nitrogen in pig muscle*

Results of Table 2 are here expressed as a percentage of the total N.

	Foetus		Piglet			Adult
	46 days	90 days	Newborn	3 weeks	4-6 weeks	
Non-protein N	13.1	17.6	19.2	12.1	15.0	12.9
Sarcoplasmic-protein N	48.2	21.0	16.2	18.7	19.0	24.3
Fibrillar-protein N	30.2	53.5	47.8	56.5	59.0	60.3
Alkali-insoluble = extracellular-protein N	4.8	8.8	16.9	11.2	7.3	3.3
Collagen N	4.8	10.5	9.6	10.3	7.0	3.7

for total N (Dickerson, 1960). The contribution of non-protein N changed very little during the development of human muscle and formed a lower proportion of the total N than in pig muscle. In the pig it showed a peak value in the muscle of the newborn animal. In both species the contribution of the sarcoplasmic proteins decreased during foetal development and then increased during post-natal life. The contribution of the fibrillar proteins scarcely changed during the foetal development of man. In the pig they increased between 46 and 90 days' gestation and then probably changed little during the remainder of the pre-natal period. In both species there was an increase during post-natal growth. In the pig most of this took place in the first 4-6 weeks, whereas in man some increase continued after 7 months. The fibrillar proteins contributed a greater proportion of the total N in adult human muscle than in that of the adult pig. The extracellular proteins reached a maximum of 17-18% of the total N in the newborn of both species, and still accounted for 15% in the muscle of the infants of 4-7 months, whereas in the pig the contribution had fallen to 11% at 3 weeks.

Water and inorganic constituents

Table 5 shows the composition of human skeletal muscle at five ages. Victims of accidents were chosen to provide the adult material because it was thought that their tissues would be more likely to be normal in composition than those of persons dying of disease. It was subsequently realized, however, that haemorrhage makes considerable difference to the composition of some of the tissues,

particularly skeletal muscle (Widdowson & Southgate, 1959), and all four men and the 11-year-old boy had suffered some haemorrhage, two men and the boy rather severely. The fetuses and babies, on the other hand, had not suffered any abnormal loss of blood. There is considerable information in the literature on the composition of normal adult human muscle obtained by biopsy (Mudge & Vislocky, 1949; Mokotoff, Ross & Leiter, 1952; Baldwin, Robinson, Zierler & Lilienthal, 1952; Talso, Spafford & Blaw, 1953; Wilson, 1955; Barnes, Gordon & Cope, 1957), though little or nothing seems to have been done in the past on immature muscle. The present results for muscle of fetuses and newborn babies are therefore considered in relation to previous workers' findings for adult muscle obtained by biopsy as well as in relation to the results for adult muscle in this investigation. The figures for the composition of the muscle of the 11- and 16-year-old boys were so similar to those for the adults that the figures have been averaged together. The effects of the haemorrhage are seen in the values for Na and Cl. The figures obtained for the two adults who sustained only slight bleeding and the boy who did not bleed at all are for most constituents in fairly good agreement with the average of values found in the literature for muscle taken from normal adults by biopsy.

Table 6 shows the corresponding values for muscle obtained from pigs at six stages of development. Four of the five adult animals were killed at the slaughterhouse and their hearts were punctured, and the fifth died under an anaesthetic and did not

Table 5. *Composition of human muscle*

Results are expressed per kg. of fresh muscle; average and range are given. Values for adult muscle are quoted from Mudge & Vislocky (1949); Baldwin, Robinson, Zierler & Lilienthal (1952); Mokotoff, Ross & Leiter (1952); Talso, Spafford & Blaw (1953); Wilson (1955); Barnes, Gordon & Cope (1957).

	Foetus		Baby		Adult		
	13-14 weeks	20-22 weeks	Newborn	4-7 months	Slight bleeding	Severe bleeding	Biopsy samples from literature
	3	4	4	3	3	3	—
No. of samples ...	3	4	4	3	3	3	—
Average body wt. (kg.)	0.043 (0.030-0.067)	0.255 (0.198-0.314)	2.72 (2.41-3.54)	7.7 (7.0-9.0)	—	—	—
Composition of muscle							
Water (g.)	907 (904-913)	887 (883-894)	804 (793-812)	785 (782-790)	760 (744-773)	765 (754-774)	792 (776-803)
Total N (g.)	11.2 (10.4-11.8)	14.8 (14.0-15.3)	20.7 (19.8-21.6)	29.1 (28.3-29.8)	32.3 (31.0-34.4)	31.0 (30.0-32.1)	30.8 (29.5-31.7)
Na (m-equiv.)	101* (52.1-60.6)	90.6 (83.2-104)	60.1 (52.0-69.8)	50.1 (43.0-63.5)	30.0 (29.0-31.0)	23.0 (22.0-24.7)	36.3 (23.7-43.6)
K (m-equiv.)	56.3 (52.1-60.6)	57.6 (53.0-62.0)	57.7 (53.0-63.7)	89.5 (80.8-94.6)	102 (98-106)	103 (91-109)	92.2 (85.6-95.2)
Cl (m-equiv.)	76.4 (71.7-81.0)	65.6 (61.1-69.2)	42.6 (38.3-46.0)	35.5 (34.0-36.6)	22.1 (19.6-24.5)	17.3 (17.0-18.0)	22.1 (14.1-25.6)
P (m-moles)	36.5 (34.2-41.5)	40.0 (38.5-41.7)	47.0 (44.0-50.5)	64.9 (63.0-67.5)	65.0 (60.0-68.0)	67.0 (65.0-68.0)	58.8 (56.0-62.0)
Mg (m-equiv.)	11.7* (6.9-13.1)	10.5 (6.9-13.1)	14.8 (11.2-18.1)	20.0 (19.0-21.9)	19.2 (16.0-19.9)	19.8 (17.7-22.7)	16.7 (16.0-16.8)
Ca (m-equiv.)	5.6* (5.5-11.9)	7.1 (5.5-11.9)	4.3 (2.2-6.6)	3.1 (2.3-3.7)	2.8 (2.2-3.3)	2.5 (1.9-2.9)	—

* One result only.

Table 6. *Composition of pig muscle*

Results are expressed per kg. of fresh muscle; average and range are given.

	Foetus		Piglet			Adult	
	46 days	90 days	Newborn	3 weeks	4-6 weeks	Not bled	Bled
No. of samples ...	5 litters	2 litters	12	2	3	1	4
Body wt. (kg.) ...	0.025 (0.012-0.045)	0.77 (0.70, 0.83)	1.21 (0.92-1.60)	3.45 (3.2, 3.7)	8.8 (6.9-12.3)	—	195 (184-208)
Composition of muscle							
Water (g.)	916 (913-919)	873 (872, 874)	823 (815-849)	778 (777, 780)	788 (785-791)	735	749 (742-756)
Total N (g.)	8.5 (8.2-8.8)	11.4 (10.7, 12.0)	15.4 (14.1-16.5)	29.1 (28.8, 29.4)	26.9 (26.2-27.5)	31.1	35.6 (34.5-36.5)
Na (m-equiv.)	106 (102-110)	77.0 (70.9, 83)	54.3 (47.1-60.6)	34.3 (33.6, 35.0)	28.5 (27.8-29.1)	24.2	19.1 (18.4-20.7)
K (m-equiv.)	46.7 (42.8-50.3)	60.4 (57.8, 62.9)	72.5 (69.0-75.6)	102 (101, 103)	108 (103-115)	92.5	102 (95-107)
Cl (m-equiv.)	72.7 (70.0-77.5)	46.0 (44.2, 47.7)	36.8 (31.5-41.2)	32.0 (30.5, 33.5)	23.5 (22.7-24.3)	21.1	13.2 (12.6-14.4)
P (m-moles)	30.7 (27.5-34.3)	45.6 (43.1, 47.5)	56.8 (51.0-60.0)	76.8 (76.8, 76.8)	76.5 (73.0-78.0)	68.0	72.5 (68.3-74.0)
Mg (m-equiv.)	9.0 (7.2-12.5)	12.2 (12.1, 12.2)	15.3 (13.9-16.6)	16.2 (13.5, 18.9)	21.9 (20.5-23.2)	23.2	21.8 (19.5-24.0)
Ca (m-equiv.)	7.3 (6.0-8.5)	3.5 (3.6, 3.4)	2.9 (2.5-3.2)	3.3 (3.3, 3.3)	2.1 (2.0-2.2)	2.3	2.1 (1.8-2.8)

bleed extensively before death. The results for this pig are presented separately, and the effect of bleeding on the inorganic constituents is once again seen. None of the younger pigs suffered any loss of blood before it died.

In both species the fall in the percentage of water during development was accompanied by a rise in total N, and this has been commented on with reference to Tables 1 and 2. The values for total N in Tables 5 and 6 are not exactly the same as the sum of the protein and non-protein N in Tables 1 and 2 because not all the muscle samples analysed for inorganic constituents were subjected to a detailed fractionation of their N, but the figures at each age are never far apart.

Fat was determined on only a few of the muscle samples analysed in this investigation. From the values of $N \times 6.25$ plus water, however, it is clear that in no case was there room for more than 2-3 % of fat, except in the newborn of both species. In newborn-pig muscle 93 % could be accounted for as protein, water and inorganic constituents. The body of the newborn pig contains only about 1 % of

fat (Widdowson, 1950), so fat is unlikely to contribute the 7 % that is missing in the muscle, and it is suggested that this is largely made up of polysaccharides. The muscle of the newborn pig has been shown to contain 7 % of glycogen (McCance & Widdowson, 1959), and although no precautions were taken to retain the glycogen in these samples of muscle, its breakdown products must still have been there. Whether the newborn human has a high concentration of glycogen in its muscle is not known.

There was a big fall in the concentration of the extracellular Na^+ and Cl^- ions with development in both species, and a rise in the intracellular constituents K and P, and with respect to its inorganic constituents the muscle of the pig at birth seemed more advanced chemically than the muscle of the full-term baby. The high concentrations of Ca in the muscle of the human and pig foetuses and the low values for Mg have already been mentioned by Economou-Mavrou & McCance (1958). Some of our material was in fact presented in their paper. Values for newborn muscle and muscle from older

Table 7. *Composition of human muscle*

Data are derived from average results given in Tables 1 and 5.

	Foetus		Baby		Adult		
	13-14 weeks	20-22 weeks	Newborn	4-7 months	Slight bleeding	Severe bleeding	Biopsy samples from literature
Chloride space (g./kg.)	672	577	350	293	183	143	183
Sodium space (g./kg.)	796	714	431	354	212	162	256
Intracellular water (g./kg.) (I.C.W.)	235	310	454	492	577	622	609
Intracellular-protein N (g./kg.) (I.C.P.N)	10.5	12.0	14.6	21.8	28.3	27.2	27.0
Intracellular K (m-equiv./kg.) (I.C.K)	49.4	51.7	54.9	87.9	101	102	91.2
Intracellular Na (m-equiv./kg.) (I.C.Na)	15.4	17.3	11.2	8.5	4.0	2.6	10.3
I.C.P.N (g./kg. of I.C.W.)	44.7	38.7	32.2	44.3	49.0	43.7	44.4
I.C.K (m-equiv./kg. of I.C.W.)	210	167	121	179	175	164	150
I.C.Na (m-equiv./kg. of I.C.W.)	65.5	55.9	24.7	17.3	6.9	4.2	16.9
Mg (m-equiv./kg. of I.C.W.)	49.8	33.9	32.6	40.7	33.2	31.8	27.4
Total cations (I.C.K + I.C.Na + Mg) (m-equiv./kg. of I.C.W.)	325	256	178	236	216	200	194
P (m-moles/kg. of I.C.W.)	155	129	104	132	113	108	97
I.C.K (m-equiv./g. of I.C.P.N)	4.71	4.31	3.76	4.02	3.57	3.75	3.38
Mg (m-equiv./g. of I.C.P.N)	1.11	0.88	1.01	0.92	0.68	0.73	0.62
Total cations (I.C.K + I.C.Na + Mg) (m-equiv./g. of I.C.P.N)	7.3	6.6	5.5	5.3	4.4	4.6	4.4
P (m-moles/g. of I.C.P.N)	3.48	3.34	3.22	2.97	2.30	2.46	2.18

babies and piglets have now been added; they lie between those for the foetus and the adult. The significance of the high concentration of Ca in foetal muscle will be discussed later. The concentration of Mg increases with development along with that of the other intracellular ions.

From the values given in Tables 3–6 calculations have been made of the relation between the cellular constituents at the various ages, in an endeavour to discover whether or not the foetal muscle cell has the same gross chemical composition as that of the adult. The results are set out in Tables 7 and 8. The 'chloride space' per kilogramme of muscle was calculated in the conventional way, from a knowledge of the concentration of Cl and protein in the serum (Widdowson & McCance, 1956), and of Cl in muscle at the various ages. The correction factor for the Gibbs–Donnan equilibrium varied according to the concentration of protein in the serum (Van Slyke, 1926), from 1.05 for the adults of both species with 7–8% of protein, to 1.014 for the 46-day-old pig foetuses with 2% of protein. The 'sodium space' was similarly calculated with a

Gibbs–Donnan factor of 0.94 for the adults and 0.983 for the 46-day-old pig foetuses. The values for 'chloride space' were subtracted from the values for total water shown in Tables 5 and 6 to obtain the 'intracellular' water. The values for intracellular water will be correct only if all the Cl in skeletal muscle is extracellular at all ages and if the Cl is uniformly distributed in the water in the extracellular phase. The extracellular phase in muscle consists of a gel containing hyaluronic acid and other mucopolysaccharides, and it also contains collagen and elastic fibres. There is evidence that the water associated with the latter contains a higher concentration of Cl than true extracellular fluid (Manery, Danielson & Hastings, 1938), but this introduces only a very small error and, although it has been found that hyaluronic acid 'binds' Cl *in vitro*, it seems that this is unlikely seriously to affect the calculation of the 'chloride space' (Aldrich, 1959). Thus even in the light of recent research it is reasonable to assume that, in adult muscle, 'chloride space' gives a fair measure of extracellular water (Fenn, 1936). The same seems

Table 8. *Composition of pig muscle*

Data are derived from average results given in Tables 2 and 6.

	Foetus		Piglet			Adult	
	46 days	90 days	Newborn	3 weeks	4–6 weeks	Unbled	Bled
Chloride space (g./kg.)	716	453	347	264	196	174	109
Sodium space (g./kg.)	866	590	383	202	196	166	131
Intracellular water (g./kg.) (I.C.W.)	200	420	476	514	592	561	640
Intracellular-protein N (g./kg.) (I.C.P.N)	6.7	8.3	9.9	22.4	21.0	26.4	30.2
Intracellular K (m-equiv./kg.) (I.C.K)	34.1	55.8	69.5	100	107	91.4	101
Intracellular Na (m-equiv./kg.) (I.C.Na)	18.2	18.9	5.0	0	0	0	3.05
I.C.P.N (g./kg. of I.C.W.)	33.5	19.8	20.8	43.6	35.4	47.0	47.1
I.C.K (m-equiv./kg. of I.C.W.)	170	133	146	194	180	163	159
I.C.Na (m-equiv./kg. of I.C.W.)	91.0	45.0	10.5	0	0	0	4.7
Mg (m-equiv./kg. of I.C.W.)	45.0	29.0	32.2	31.5	36.9	41.3	34.0
Total cations (I.C.K + I.C.Na + Mg) (m-equiv./kg. of I.C.W.)	306	206	189	230	218	204	197
P (m-moles/kg. of I.C.W.)	154	108	119	147	128	121	113
I.C.K (m-equiv./g. of I.C.P.N)	5.10	6.72	7.02	4.46	5.10	3.46	3.36
Mg (m-equiv./g. of I.C.P.N)	1.35	1.46	1.55	0.72	1.04	0.88	0.72
Total cations (I.C.K + I.C.Na + Mg) (m-equiv./g. of I.C.P.N)	9.2	10.4	9.1	5.2	6.1	4.4	4.1
P (m-moles/g. of I.C.P.N)	4.60	5.47	5.74	3.42	3.63	2.56	2.39

to be true of muscle from newborn pigs (Widdowson & Southgate, 1959). There is up until now no proof about this one way or the other in foetal muscle.

The intracellular fluid consists of a solution of inorganic substances, of which K, Mg and P are quantitatively the most important. It must also contain some, if not the greater part, of the non-protein N measured and shown in Tables 1 and 2, which consists mainly of purines, amino acids, creatine and urea. For reasons given above it seems more logical, in trying to find out whether the foetal cell contains more fluid in proportion to solid matter than the adult cell, to consider the relation between the intracellular fluid and the intracellular-protein N, rather than the whole of the non-collagen N as suggested by Lilienthal, Zierler, Folk, Buka & Riley (1950). Intracellular-protein N has accordingly been used as the basis of the calculations.

Widdowson & McCance (1956) reported that foetal extracellular fluids have a higher concentration of K than those of the adult. More recently it has been suggested (S. James, 1958, personal communication) that the high values found may not be the physiological ones but may have been due to asphyxia, which is the explanation put forward by some authors for the high serum K sometimes found in the human at birth. Be that as it may, it does not alter the fact that the foetus has a larger volume of extracellular fluid in proportion to its weight than the adult, so that, unless the concentration of K in it is actually lower than in the extracellular fluid of the adult, more of the K in foetal tissues must be extracellular. The extracellular K in the muscle at each age had been calculated from the chloride space and the concentration of K in the extracellular fluid, assuming the values of Widdowson & McCance (1956) to be correct, and deducted from the values for total K shown in Tables 5 and 6. This gave the concentration of intracellular K in the muscle. Similarly, the Na accommodated in the extracellular fluid has been calculated and subtracted from the total Na in the muscle to give the intracellular Na. The proportion of the total P and Mg that is extracellular is so small even in the foetus that no correction has been applied for this in the calculations on which Tables 7 and 8 are based. It should be noted that the fact that no correction has been made for the small amount of fat in muscle makes no difference to the relationships set out in the Tables.

Development in both species was accompanied by a fall in the proportion of the tissue occupied by the 'chloride space', and an increase in the proportion of intracellular water. The 'sodium space' was always larger than the 'chloride space' in human muscle; in pig muscle the 'sodium spaces' were larger at the younger ages but not in the older ones. This suggests that the foetal-muscle cells

contained Na, and the concentrations of Na in the intracellular water are shown in Tables 7 and 8.

The amounts of intracellular K, P and Mg per litre of intracellular water agree well with values reported in the literature for adult human muscle (Mokotoff *et al.* 1952; Talso *et al.* 1953; Horvath, Berg, Cummings & Shy, 1955; Wilson, 1955). Values for intracellular protein N are now added. The intracellular relationships in mature pig muscle are similar to those in human muscle, and other workers have emphasized the constancy in this aspect of chemical anatomy from one species to another (Harrison & Darrow, 1938).

The muscle of the newborn of both species appears to have less N and also less K and P and less total cations per litre of intracellular water than adult muscle, and the same also applied to the pig foetuses that were not far from term. In the younger foetuses the concentrations were higher. If the concentration of K in foetal extracellular fluid had been the same as that in the adult the concentration in the intracellular water of the 46-day-old pig foetuses would have been higher still. These high values for N, P and cations in the younger foetuses might be interpreted as showing that foetal-muscle cells contain a higher proportion of protein in relation to fluid, and that the fluid has a higher concentration of inorganic ions than the muscle cells at birth, but it might equally well be taken as a warning that the value for intracellular water in the foetal muscle is wrong because Cl is not entirely extracellular. If some of the Cl were inside the cells, then the true extracellular-fluid volume would be smaller than the 'chloride space', and the volume of intracellular fluid calculated from the 'chloride space' would be too small, and the protein and inorganic constituents too high in relation to it.

The intracellular K, P, Mg and total cations have also been calculated per gram of intracellular-protein N, a relationship which depends to a much smaller extent on the assumption that Cl is all extracellular. There are minor fluctuations for the pig, but the generalization is true that the adult muscle of both species contained less intracellular inorganic constituents in proportion to intracellular protein than the muscle of the foetus. A possible interpretation of these findings is given below.

It should be noted how little effect haemorrhage seems to have had on the muscle cell. This has been discussed by Widdowson & Southgate (1959).

DISCUSSION

The changes in the proportion of the various protein fractions are on the whole very similar to those found by Robinson (1952*a*) in developing chick

Fig. 1. Transverse sections of human middle quadriceps muscle. Stained with haematoxylin and eosin; magnification $\times 370$. (a) Foetus, 20 weeks' gestation; (b) newborn baby at term; (c) adult.

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Fig. 2. Transverse sections of pig middle quadriceps muscle. (a)–(c) Stained with haematoxylin and eosin; magnification $\times 390$. (d) Stained by Gordon & Sweets' silver-impregnation method for reticulin; magnification $\times 87$. (a) Foetus, 46 days' gestation; (b) newborn piglet; (c) adult; (d) foetus, 46 days' gestation.

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muscle, and these have also been confirmed at and after hatching (Dickerson, 1960). In the two mammalian species, as well as in the chick, the proportion of the total nitrogen contributed by the extracellular fraction increases to a maximum and then decreases to a lower level in the adult. The extracellular proteins are distributed in skeletal muscle as the endomysium round the individual fibres, the perimysium round the fasciculi and the epimysium round the bundles of fasciculi which constitute the entire muscle. Figs. 1 and 2 show the histological appearance of foetal, newborn and adult muscle of man and the pig respectively. In the muscle of the 20-week-old human foetus the fibres are small, relatively few in number and widely separated by extracellular material. The nuclei occupy a larger proportion of the cell than they do at the later ages. At term, the fibres are still small, but there are many more of them and they are more closely packed together. In adult muscle the fibres are very much larger in diameter, the nuclei are comparatively small and there is little space for extracellular material between the fibres. The pig foetus at 46 days is at an earlier stage of development than the human foetus at 20 weeks. The muscle fibres, or myotubes as they may be called at 46 days' gestation in the pig, are few, and they constitute only part of the total cellular material in the tissue. The other cells appear to be fibroblasts and these are scattered throughout the tissue surrounding the myotubes. The spaces between the cells are no doubt filled with the ground substance of the extracellular phase containing fine reticulin fibres (see Fig. 2*d*). Between 46 days and the end of gestation there is a large increase in the number of fibres and these are now arranged in bundles. There is, however, very little difference in the diameter of the fibres at this stage, and the nuclei are still relatively large, though now at the periphery of the cell. The adult pig muscle is very similar in appearance to that of adult man.

In the development of human muscle the number of fibres increases during about the first 6 months of gestation, but MacCallum (1898) found no evidence of increase in the number of fibres in the sartorius muscle after this age, and subsequent 'growth' was found to be 'hypertrophy' of the existing fibres. Similarly, McMeekan (1940) found no evidence of an increase in the number of muscle fibres after birth in the pig. Thus, broadly speaking, the increase in the proportion of extracellular protein takes place whilst the cells are increasing in number, but a decrease takes place whilst they are increasing in size. It is suggested that the lag in the decrease of the proportion of these proteins after birth may be due to thickening of one or all of the connective-tissue layers.

If the appearance of the sections of the muscle of the newborn of the two species is compared (Figs. 1*b*, 2*b*) it will be seen that the pig muscle appears much less mature than that of the human baby. This difference in appearance is possibly an artifact due to the fixation of the tissue in formalin-0.9% sodium chloride (1:9, v/v). There was an obvious difference between the behaviour of the muscle of the newborn of the two species when placed in this fixative, for as soon as newborn piglet muscle was put into it a white cloudiness appeared which precipitated on standing. Preliminary analyses have shown that this precipitate contains nitrogen and its chemical nature is being further investigated.

Development is associated with changes in the relative proportions of sarcoplasmic and fibrillar proteins in the muscle cells. The rate at which the fibrillar proteins develop may be influenced by the functional activity of the muscle. In the pig it was found that this fraction almost reached its adult proportion of the total nitrogen during the first 4-6 weeks after birth, whereas in man there was a smaller increase in the first 5-7 months of post-natal life. This species difference may be attributed to the fact that pigs run about soon after they are born, whereas babies do not walk until after they are 7 months old. Chicks are active as soon as they leave the egg, and a still more rapid increase in fibrillar proteins was found in this species (Dickerson, 1960).

The idea of 'chemical maturity' of an animal was introduced by Moulton (1923). Spray & Widdowson (1951) emphasized that this term can only be rightly applied to the body when all its constituents have reached adult proportions. For skeletal muscle, the results of the present investigation show that chemical maturity is reached sometime after 7 months in man and after 6 weeks in the pig. The assessment of the degree of chemical maturity of muscle at corresponding ages is difficult. In some respects human muscle at birth, for instance, is chemically more advanced than that of the newborn piglet, whereas in other respects the reverse is true. The higher concentration of potassium and phosphorus in the muscle of the newborn piglet may, however, be associated with the presence of the large amounts of glycogen which are known to be present, for both these constituents move into cells during the formation of glycogen (Fenn, 1939), and thus they may have nothing to do with 'chemical maturity' in its usual sense.

Fenn (1939) found that water also moves into cells during the deposition of glycogen, and this would therefore result in a dilution of the proteins and account for the finding of a low ratio of protein nitrogen to cell water at and near term in piglet muscle.

Information obtained about the composition of

cells from analyses of whole tissues is of necessity an average of all the different kinds of cells present in the sample. In adult or even in newborn muscle the muscle cells constitute almost all of the total cell mass. At earlier ages this may not be so. The large number of fibroblasts in the muscle of the 46-day-old pig foetus must have made a significant contribution to the cell mass of the muscle. Caution must therefore be exercised in the interpretation of the results at this age in terms of the composition of the muscle cell.

The large nuclei in foetal-muscle cells must mean that there is a high nuclear-cytoplasmic ratio as compared with more mature muscle cells. It may therefore be assumed that the composition of the nucleus will have a greater influence on the composition of the cell in the foetus than in the newborn, and greater still than in the adult. Robinson (1952*b*) found this to be true in chick muscle, for in embryos weighing about 10 g. as much as 66% of the nitrogen in his fibrillar fraction was contributed by nucleoprotein. The contribution of the nuclei to the protein fractions has not been studied in the present investigations, but it can reasonably be assumed that nucleoproteins will contribute more to the cellular nitrogen in foetal than in adult muscle. In making the calculations on the composition of the cells no account has been taken of the possibility that the nuclei might be of different composition from the cytoplasm. If this is so, then the explanation of some of the findings during the early stages of development may be that they are but a reflexion of changes in the proportion of nuclear to cytoplasmic material in the cells. There is so far very little information about the composition of the different cellular inclusions, and what there is has mostly been obtained on material other than skeletal muscle (Itoh & Schwartz, 1956). Scott (1932) did, however, show that adult muscle nuclei contain significantly higher amounts of calcium than most of the surrounding cytoplasm, and Scott & Packer (1939) showed that there was some localization of calcium at the contraction nodes. A decrease in the proportion of nucleus to cytoplasm thus seems to be a possible explanation of the decrease in calcium concentration in skeletal muscle with development. Hines & Knowlton (1933) suggested a similar explanation for the high calcium content of muscle in denervation atrophy.

The composition of muscle nuclei is being investigated and when more is known about their composition it may be possible to give a fuller interpretation of the results of this investigation.

SUMMARY

1. Skeletal muscle from man and the pig has been analysed for water, inorganic and nitrogenous

constituents at various stages of development before and after birth. The nitrogen has been divided into four fractions: non-protein nitrogen, sarcoplasmic-protein nitrogen, fibrillar-protein nitrogen and extracellular-protein nitrogen.

2. There was an increase in the concentration of the cellular constituents, nitrogen, potassium, phosphorus and magnesium with development, and a decrease in the concentration of the extracellular ions sodium and chloride. There was also a decrease in the concentration of calcium.

3. The concentration of non-protein nitrogen increased in the early stages of development but reached its adult level soon after birth. There was little change in the concentration of sarcoplasmic protein before birth, but an increase after. The biggest change took place in the fibrillar fraction, which increased in concentration throughout development. The highest concentration of extracellular protein nitrogen was found in the muscle of babies and piglets during the suckling period.

4. An attempt has been made to discover whether the muscle cell of the foetus has the same chemical composition as that of the adult, and the chemical findings have been related to the histological appearance of the muscle at the different ages.

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Aromatic-Alcohol-Oxidase Activity in the Growth Medium of *Polystictus versicolor*

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To gain a better understanding of the changes which plant lignins undergo in the soil the fungal breakdown of aromatic compounds related to lignin has been studied. Previous work has shown that the wood-rotting basidiomycete *Polystictus versicolor* is able to reduce many aromatic acids to the corresponding alcohols, or to mixtures of aldehydes and alcohols (Farmer, Henderson & Russell, 1959). The persistence of aldehydes after complete reduction of the acids appeared to be due to an extracellular oxidase acting on the alcohols, and this was confirmed for *p*-methoxybenzyl alcohol. The properties and substrate specificity of this system are more fully examined here.

MATERIALS AND METHODS

Organism. *Polystictus versicolor* as used previously (Farmer *et al.* 1959) was isolated from fructifications collected from a tree stump.

Buffers. Sørensen's phosphate (Clark, 1928) and 2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer (Gomori, 1955) were used.

Production of enzyme. The fungus was grown in 250 ml. conical flasks containing 100 ml. of mineral salts solution (NaNO₃, 0.3%; MgSO₄, 0.05%; KH₂PO₄, 0.1%; KCl, 0.05%) to which were added 1.0 g. of glucose and 0.5 g. of

yeast extract. Each flask was inoculated with four disks, cut from the periphery of a culture of the fungus on potato-dextrose-agar, and incubated at 22°, usually for 10–12 days. Hyphae from these disks spread over the surface of the medium to form a firm mat. During growth of the fungus the enzyme accumulated in the growth medium, and this solution was used directly in many experiments. A further supply of enzyme could be obtained by pouring off the medium at the end of the growth period, washing the mats three times with sterile water and then pouring 100 ml. of sterile water under them. After further incubation enzyme diffused from the mycelium into the water.

An enzyme concentrate was obtained by saturating growth solutions with (NH₄)₂SO₄. The saturated solution was allowed to stand overnight and the resulting precipitate was centrifuged down at 38 000 *g* for 20 min. It was then suspended in 3–5 ml. of water and dialysed for 21 hr. against Sørensen's phosphate buffer (KH₂PO₄–Na₂HPO₄, pH 6.2) at 5°. Under these conditions the volume did not change appreciably.

Enzyme activity. The activities of enzyme solutions were compared spectrophotometrically by measuring the rates at which they oxidized *p*-methoxybenzyl alcohol to the aldehyde, and are reported as μ moles of aldehyde produced by 1 ml. of enzyme solution in 1 hr. (μ moles/ml./hr.). The assay solution contained 1 ml. of 0.067 *M*-phosphate buffer (pH 6.2), 4 μ moles of *p*-methoxybenzyl alcohol, *x* ml. of enzyme solution and water to 3 ml. The change in absorption per minute ($\Delta E/\text{min.}$) was measured at 290 *m* μ in