the chick have been presented with reference both to the fresh weight of the tissue and the total protein. Protein analyses of the muscles of month-old and newly hatched chicks are contrasted with those of adult muscle from the rabbit.

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Changes in the Nucleoprotein Content of Chick Muscle during Development

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Whilst adult striated muscle is particularly well adapted to perform its specific function in the body, it nevertheless possesses certain elements, such as the nuclei and the particulate components of the cytoplasm, which are common to all cells. The quantitative contribution of these latter to the whole varies during development; it is large in the earlier embryonic stages, but falls, as the myofibrils differentiate, to a lower level in the adult muscle (Graff & Barth, 1938; Caspersson, 1941, 1947, 1950; Brachet, 1945, 1947).

In a previous paper a protein fractionation procedure has been described and applied to chick muscle at various developmental stages (Robinson, 1952). Since the nucleoproteins of the cell nucleus have solubility properties similar to those of the myofibrillar proteins (Brachet & Jeener, 1947; Mirsky & Pollister, 1942, 1943), they have been estimated with them in this scheme. Thus, whilst none of the deoxyribonucleic acid of the cell nucleus was found in dilute KCl-phosphate extracts of muscle (Fraction 1) over 80% was extracted in

strong KCl-phosphate solutions and was precipitated, with the myofibrillar proteins, by 15% (v/v) ethanol on dilution to an ionic strength of 0·12 at 0° (Fraction 2). The remaining 20% of the deoxyribonucleic acid was not extracted and was estimated in the residual fractions (Fractions 3 and 4). The numerical designations of the fractions refer throughout this paper to Robinson (1952).

In this study the contribution made by deoxyribonucleoproteins of the cell nucleus has been assessed as follows. Nuclei have been isolated from chick muscle at different developmental stages and the deoxyribonucleic acid/protein ratio has been determined. Then, knowing the deoxyribonucleic acid content of the muscle residue after extraction with dilute KCl-phosphate solution (Fraction 2 + 3 + 4), the amount of deoxyribonucleoprotein in this fraction has been calculated. Furthermore, a better assessment of the myofibrillar protein content of muscle has been made by correcting the previous analyses for the newly determined nuclear protein contribution.

EXPERIMENTAL

The sources of muscle material and details of the dissection and extraction techniques have been given previously (Robinson, 1952). The nucleic acid contents of the following fractions have been determined; Fraction 1, the extract of muscle in 0.1 M-KCl, 0.066 M-NaH$_2$PO$_4$/K$_2$HPO$_4$, $I=0.2$, pH 7.1, and Fraction $2+3+4$, the residue from the extraction of muscle with the above solution.

The terms deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) are used as convenient notations without necessarily assuming that the nucleic acid material from the muscle is identical with that from other organs.

Extraction and estimation of nucleic acids. Schneider's (1945) procedure for the extraction of nucleic acids was used without serious modification. Total nucleic acids were estimated from their purine and pyrimidine absorption in the ultraviolet, measured in the Beckman spectrophotometer (model DU), and from their organic phosphorus content determined by Allen's (1940) method. DNA was determined by a modification of the Dische (1930) method described by Davidson & Waymouth (1944), and RNA was estimated by a modification of the orcinol method of Mejbaum (1939) due to Militzer (1946), but using the period of heating recommended by Albaum & Umbreit (1947). The orcinol estimations were always corrected for the effect of any DNA which was present.

The standard nucleic acids used were kindly provided by Dr K. Bailey. The RNA was a Boehringer product from yeast and the DNA a pure sample from thymus gland. Both preparations were free from inorganic phosphorus and contained 9.60 and 9.63% organic phosphorus respectively. After treatment with hot trichloroacetic acid, as in the Schneider (1945) procedure, the ultraviolet absorption maximum of the DNA standard solution, measured against a blank solution of trichloroacetic acid, is displaced from 2600 to 2680 A. Using the notation suggested by Chargaff & Zamenhoff (1948) the atomic extinction coefficients (ϵP) for our acid-treated samples were 10 050 for RNA and 8450 for the DNA sample. The DNA value is considerably higher than that given by Vischer & Chargaff (1948) (9800 and 6056 respectively), but both are in good agreement with values quoted by Ogur & Rosen (1950) (10 800 and 8780 respectively) for samples after treatment with perchloric acid. In addition to causing the shift in maximal absorption it appears that breakdown of DNA to nucleotide units by acid treatment increases the degree of absorption in the ultraviolet.

The nucleic acid extracts were diluted to contain 10–20 μg. nucleic acid/ml. for measurements of ultraviolet absorption; 10–60 μg. nucleic acid/ml. for measurements of phosphorus content; 50–200 μg. nucleic acid/ml. for estimations by the orcinol method for RNA; 500–1500 μg. nucleic acid/ml. for estimations by the diphenylamine method for DNA.

The sum of the separate analyses for DNA and RNA in each fraction should agree with the total nucleic acid values derived from the ultraviolet absorption and the phosphorus determination; although there was reasonable agreement for the extract of muscle in dilute KCl-phosphate (Table 1), the sum was in general slightly higher in the analyses of the residue (Table 2), due possibly to the presence of mucopolysaccharide material in this fraction.

Since the only nucleic acid present in the dilute KCl-phosphate extract is RNA the ultraviolet extinction coefficient of the RNA standard was used to calculate the nucleic acid content of this fraction. In the residue, in view of the preponderance of DNA, the corresponding coefficient of the DNA standard was used.

Isolation of muscle nuclei. Nuclei were isolated at pH 4 using a citric acid method similar to that described by Dounce (1943). Differential centrifugation of the homogenized muscle (4–10 g.) yielded samples of nuclei free from sarcoplasmic and fibrillar material when examined under the microscope (Fig. 1). The nuclei seem to have retained their
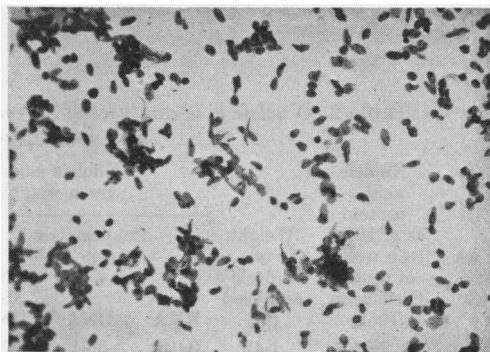

Fig. 1. Preparation of cell nuclei from muscle of embryos incubated for 16 days. Stained with haematoxylin and eosin. Magnification, $\times 370$.

shape, appear unbroken, and in certain cases nucleoli are visible. In the adult animal, where the nuclei form a very small part of the whole muscle, greater difficulty was encountered in obtaining a preparation free from fibrillar fragments. Nevertheless, this could be achieved by using larger quantities of starting material and by sacrificing a greater proportion of the contaminated nuclei.

The amount of citric acid required to maintain the pH around 4 varied according to the state of development of the tissue and had to be determined previously by titration. Small quantities were added at intervals throughout the preparation to maintain the pH, which otherwise tended to rise and cause agglutination.

It is probable that nuclei isolated using such a method will have lost a considerable amount of protein during their preparation. Although neither nucleic acid nor the basic histone protein is likely to be soluble at pH 4, any acidic proteins of the nucleus may be (Behrens, 1932; Dounce, 1950). However, the analyses are to be referred to the residue from the extraction of the muscle with dilute KCl-phosphate and this fraction will contain nuclei subjected similarly to aqueous media. It has been assumed that the loss of water-soluble proteins is approximately equivalent in each case, although the conditions do differ particularly with respect to pH.

Nucleic acids were extracted and estimated as before. Protein nitrogen was estimated in the residue by the micro-Kjeldahl method after the nucleic acid had been extracted. No phosphorus estimations were made.

Table 1. *Nucleic acid analyses of dilute KCl-phosphate extracts of chick muscle*

Av. wt. of chick (g.)	Nucleic acid + protein as g./100 g. fresh weight of muscle (Robinson, 1952)	Weight of muscle analysed (g.)	Nucleic acid as g./100 g. fresh weight of muscle				Nucleic acid as % of the total protein + nucleic acid of muscle			
			Colorimetric		Phosphorus Total nucleic acid	Ultra-violet Total nucleic acid	Colorimetric		Phosphorus Total nucleic acid	Ultra-violet Total nucleic acid
			RNA	DNA			RNA	DNA		
3·5	2·75	1·42	0·29	—	—	0·30	11·30	—	—	11·50
7·8	3·75	3·99	0·23	—	0·27	0·22	6·18	—	7·25	5·80
8·9	3·95	5·69	0·23	—	—	0·22	5·75	—	—	5·66
13·7	5·10	6·66	0·28	—	—	0·24	5·45	—	—	4·65
16·7	5·63	8·06	0·21	—	—	0·22	3·78	—	—	3·85
23·8	7·50	4·42	0·23	—	0·24	0·22	3·02	—	3·18	2·99
Hatching 142	18·0	7·14	0·06	—	—	0·08	0·32	—	—	0·45

Table 2. *Nucleic acid analyses of the residue remaining after extraction of chick muscle with dilute KCl-phosphate*

Av. wt. of chick (g.)	Nucleic acid + protein as g./100 g. fresh weight of muscle (Robinson, 1952)	Weight of muscle analysed (g.)	Nucleic acid as g./100 g. fresh weight of muscle					Nucleic acid as % of the total protein + nucleic acid of muscle				
			Colorimetric			Phosphorus Total nucleic acid	Ultra-violet Total nucleic acid	Colorimetric			Phosphorus Total nucleic acid	Ultra-violet Total nucleic acid
			RNA	DNA	RNA + DNA			RNA	DNA	RNA + DNA		
3·5	2·60	1·42	0·040	0·21	0·250	—	0·23	1·40	8·1	9·5	—	8·80
7·8	3·75	3·99	0·050	0·19	0·240	0·23	0·21	1·32	5·1	6·42	6·25	5·55
8·9	3·95	5·69	0·025	0·20	0·225	0·18	0·22	0·63	5·1	5·73	4·62	5·52
13·7	5·10	6·66	0·023	0·20	0·223	—	0·22	0·45	4·0	4·45	—	4·27
16·7	5·63	8·06	0·033	0·14	0·173	0·17	0·19	0·59	2·5	3·09	3·04	3·41
23·8	7·50	4·42	0·046	0·22	0·266	0·23	0·21	0·61	3·0	3·61	3·10	2·85
Hatching 142	18·0	7·14	0·039	0·033	0·072	—	0·06	0·22	0·18	0·40	—	0·34

RESULTS

Nucleic acid content of extracts and residues

The nucleic acid analyses of the dilute potassium chloride-phosphate extract are given in Table 1, and the analyses of the residue after such extraction, in Table 2.

Expressed as g./100 g. fresh weight of muscle the amounts of nucleic acid remain at a fairly constant level both in the sarcoplasmic extract and in the residue throughout embryonic development. The relative constancy of the nucleic acid content for such a long period is rather surprising when it is considered in relation to the changing composition of embryonic muscle as a whole. Comparison with the analysis of muscle from a 3-week-old chick weighing 142 g. shows that a large fall in the amount of nucleic acid/g. of muscle occurs after hatching.

When the results are expressed as percentages of the total protein of the muscle (actually protein plus nucleic acid) on the basis of the analyses previously reported (Robinson, 1952), the nucleic acids in each fraction form a steadily decreasing percentage throughout embryonic development. Novikoff & Potter (1948), in a study of the nucleic acid content of the whole chick embryo during development, find a peak in the RNA and DNA content after 15 days incubation (12 g. embryos) expressed as mg./100 g. of dry tissue. The present results show that this cannot be due to changes in the nucleic acid content of the muscle tissue.

In the embryonic muscle the RNA/DNA ratio lies between 1·6 and 1·25, while in the 142 g. chick (3 weeks old) it is approximately 3·0. Davidson & Waymouth (1944), in a study of sheep muscle, find a RNA/DNA ratio equal to 1·4 in the embryo, rising to 3·6 in the adult.

Analyses of muscle nuclei

The amount of nucleic acid, expressed as a percentage of the nucleic acid plus protein, varied between 25 and 44% at different embryonic stages for seven samples of isolated nuclei. The variations may be the result of contamination or of the extraction of varying amounts of nucleoprotein during the isolation. Analyses in the literature for the isolated nuclei of other tissues vary widely between

20 and 60% (Mirsky & Pollister, 1942; Dounce, 1943; Mirsky & Ris, 1949). The RNA/DNA ratio varied between 0·1 and 0·2, in good agreement with values in the literature (Caspersson, 1941, 1947, 1950; Davidson & Waymouth, 1944; Vendrely & Vendrely, 1948).

The nucleoprotein and myofibrillar protein content of the residue after extraction with dilute potassium chloride-phosphate

Nucleoprotein content. Using the ultraviolet absorption data of Table 2, and taking an average value of 0·33 for the ratio nucleic acid/nucleic acid plus protein in the embryonic nucleus, the changes

This has been done in the curves *B* of Figs. 2 and 3.

Myofibrillar protein content. In a previous paper (Robinson, 1952) the composition of various protein fractions of chick embryonic muscle has been discussed. It has been concluded that a strong potassium chloride-phosphate extract will contain the majority of the myofibrillar and nuclear proteins of the muscle and that these will be precipitated together on dilution with addition of ethanol (Fraction 2). A small percentage will, however, remain behind in the residue and will be extracted from this, together with other proteins, by 0·1N-sodium hydroxide (Fraction 3). The changes during
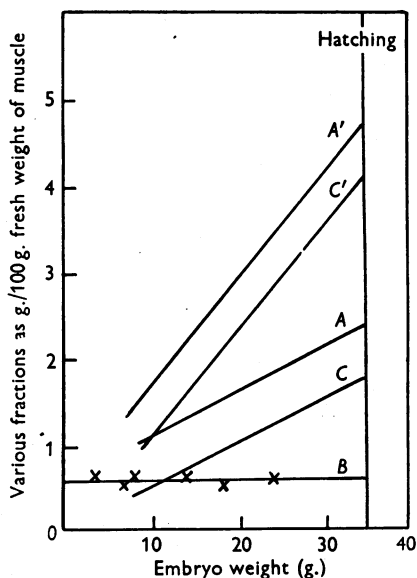

Fig. 2. Changes in the total protein, nucleoprotein and myofibrillar protein content of certain muscle fractions during embryonic development; expressed as g./100 g. fresh weight of muscle. *A*, total protein soluble in strong KCl-phosphate and precipitated by dilution and addition of ethanol (curve from data of Robinson, 1952). *A'*, total protein soluble in strong KCl-phosphate and precipitated by dilution and addition of ethanol plus that extracted from the residue by 0·1N-NaOH (curve from data of Robinson, 1952). *B*, nucleoprotein content of the muscle residue after extraction with dilute salt solutions. *C*, resultant curves obtained by subtraction of the nucleoprotein contribution (curve *B*) from the total protein of curve *A*. *C'*, resultant curve obtained by subtraction of the nucleoprotein contribution (curve *B*) from the total protein of curve *A'*.
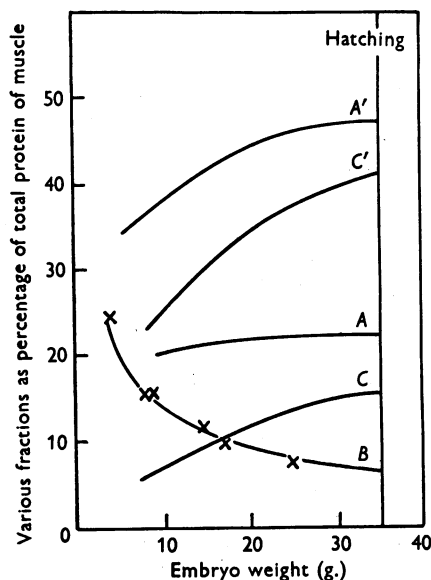
Fig. 3. Changes in the total protein, nucleoprotein and myofibrillar protein content of certain muscle fractions during embryonic development; expressed as a percentage of the total muscle protein. *A*, total protein soluble in strong KCl-phosphate and precipitated by dilution and addition of ethanol (curve from data of Robinson, 1952). *A'*, total protein soluble in strong KCl-phosphate and precipitated by dilution and addition of ethanol plus that extracted from the residue by 0·1N-NaOH (curve from data of Robinson, 1952). *B*, nucleoprotein content of the muscle residue after extraction with dilute salt solutions. *C*, resultant curve obtained by subtraction of the nucleoprotein contribution (curve *B*) from the total protein of curve *A*. *C'*, resultant curve obtained by subtraction of the nucleoprotein contribution (curve *B*) from the total protein of curve *A'*.

in the nucleoprotein content of the residue after extraction with dilute KCl-phosphate solutions can be represented in relation to the fresh weight and total protein plus nucleic acid content of the muscle (latter data derived from values in Robinson, 1952).

development in the total protein content of Fraction 2 and of Fractions 2 + 3 are represented by curves *A* and *A'* respectively in Figs. 2 and 3 (derived from the data of Robinson, 1952).

By subtracting the nucleoprotein contribution to these fractions we can therefore estimate the true myofibrillar protein content. This has been done for the proteins soluble in strong potassium chloride-phosphate and precipitated by ethanol on dilution in curve C (Fraction 2), and for these proteins plus those extracted from the residue by $0.1N$-sodium hydroxide in curve C' (Fraction 2 + 3).

The latter correction is the more justified since the whole of the deoxyribonucleoprotein is present in Fractions 2 + 3, whereas only 80 % is present in Fraction 2 alone. However, because of the greater complexity of Fraction 3, which, particularly in the early embryonic stages, is likely to include material other than that derived from the myofibrils and the nuclei, this estimate of the amount of myofibillar proteins is probably too high. Fraction 2, on the other hand, consists solely of myofibrillar and nuclear proteins and the corrected curve C, therefore, should represent more closely the changes in the myofibrillar protein content.

After correction for the deoxyribonucleoprotein, the resultant curve for the true myofibrillar protein contribution falls away towards the beginning of the incubation period. Thus the large increase in the percentage of myofibrillar protein which occurs during development becomes apparent and the curves, A and A', are seen to be the resultants of the decreasing nucleoprotein content and the increasing myofibrillar protein content.

DISCUSSION

It is clear that throughout the period of embryonic development the nucleoprotein content of the strong potassium chloride-phosphate extract of muscle is very high. It appears that at the earliest stages very little of the protein precipitated in Fraction 2 can be truly myofibrillar, if we acknowledge that the analyses of the nuclei are even approximately correct. Even at the 14-day incubation stage, when the average embryo weight is approximately 10 g., the nucleoprotein amounts to 66 % of the total protein of this fibrillar fraction. In fact, the myofibrillar protein forms only 7 % of the total protein of the muscle at this stage.

Despite the preponderance of the nucleoproteins

in these early stages, the breast muscle will respond to electrical stimulation after incubation for 8 days. The exact relation of such a contraction to the organized activity of the adult myofibril is not clear but it appears that, regardless of the great differences in the relative composition, the same contractile organization is present and functional.

Hermann & Nicholas (1949) studied the nucleic acid distribution in developing rat muscle. Under their conditions of fractionation the majority of the DNA remains unextracted, and they claim that it does not interfere with their estimations of the fibrillar proteins. However, it is likely that a considerable proportion of the myofibrillar proteins themselves must similarly remain unextracted under their conditions. It is difficult to draw comparisons between the two analyses; not only do the experimental procedures differ, but species differences may also be significant. Nevertheless, the total nucleic acid content and the DNA/RNA ratio seem to be of the same order at corresponding developmental stages.

SUMMARY

1. An assessment has been made of the nucleoprotein content of certain protein fractions of chick breast muscle in the prehatching period.

2. Nucleic acid analyses of these fractions, and nucleic acid and protein analyses of isolated muscle nuclei, have been made. From these determinations the nucleoprotein content of the fraction which contains the proteins of the myofibril has been calculated.

3. A better measure of the changes in the myofibrillar protein content has been obtained by correcting previous analyses for the nucleoprotein contribution. The large increase which occurs during embryonic development in the percentage of myofibrillar protein is thus made apparent.

4. The low myofibrillar and high nuclear protein content of muscles which, nevertheless, respond to electrical stimulation has been commented upon.

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A Study of the Adenosinetriphosphatase Activity of Developing Chick Muscle

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In 1939, Engelhardt & Lyubimova (1939) first made the important observation that a close association exists between the enzyme adenosinetriphosphatase (ATP-ase) and the myofibrillar protein, myosin, and they tentatively suggested that the enzyme might in fact form part of the myosin molecule itself. Since that time their observations have been amply confirmed by other workers. Moreover, the significance of the connecting link which is thus provided between the chemical events during muscle contraction and the protein systems presumed responsible for the mechanical changes has been widely appreciated. The muscle ATP-ase studied by Kielley & Meyerhof (1948, 1950) appears to be a distinct enzyme present in water extracts, and associated with the cell particulate bodies rather than with the myofibrils (Perry, 1952).

A study of the muscle ATP-ase during the early stages of development of the chick was begun in conjunction with other work on the developing tissue (Robinson, 1952a, b). The homogenized muscle was extracted with 0·6M-potassium chloride in 0·067M-borate buffer at pH 7. A sample of the extract was diluted to 0·05M-potassium chloride, when the myofibrillar proteins precipitated, and could be centrifuged down. The enzyme activity was determined in the whole extract, in the precipitate on dilution and in the residue left after extraction with the 0·6M-potassium chloride solution. The presence of a high proportion of the total ATP-ase activity in water extracts from muscle early in embryonic development led to a further more detailed study of such extracts.

EXPERIMENTAL

Most tissues possess a variety of enzymes whose action results in the liberation of inorganic phosphate. Thus, alkaline and acid phosphatases, pyro-phosphatases and myokinase might interfere with any attempt to estimate a specific ATP-ase activity, and it is necessary to try to define conditions for an ATP-ase estimation which will minimize any interference by such systems.

In view of the importance of the myosin ATP-ase system, many workers have investigated its properties. Not all the results are in agreement, particularly with respect to the pH optimum and the effect of ions upon the activity. However, Mommaerts & Seraidarian (1947) have been able to account for most of the differences in terms of the extreme sensitivity of the myosin ATP-ase towards electrolytes, and to show that, provided the ionic conditions are strictly controlled, it is possible to develop procedures for the specific assay of myosin ATP-ase. It is activated by calcium ions at low potassium chloride concentrations and has its maximum activity around pH 9, though the behaviour is in general too complex to be described in terms of a constant pH optimum. Myokinase will have little effect on the initial velocity if this is measured in the presence of excess substrate; moreover, the specificity of a preparation and its freedom from alkaline and acid phosphatases and from pyro-phosphatases can readily be tested.

It is more difficult to define conditions under which the Kielley & Meyerhof (1948, 1950) ATP-ase of muscle will not be estimated. This enzyme is, however, relatively unstable in the absence of cyanide ions and has a maximum activity in the presence of magnesium ions at pH 6·8: the test conditions to be described are such as to minimize its contribution to the activity measurements.

Preparation of adenosinetriphosphate. The barium salt of adenosinetriphosphate (ATP) was prepared by a method similar to that described by Needham (1942) by Mr E. J. Morgan. It was converted to the sodium salt as recommended by Bailey (1942). The salt solution was freed from