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In vitro Synthesis of γM-like Globulin by Various Chick Embryonic Cells*

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Summary. Mass cultures of cells derived from bursa of Fabricius, yolk sac and thigh muscle of 16-day chick embryos, and from the peripheral blood of adult chickens, and cloned chick embryonic muscle cells synthesize and release into the nutrient medium a protein which has the antigenic and physico-chemical characteristics of chicken γM-globulin.

INTRODUCTION

Macroglobulin antibody (γM) with a sedimentation coefficient of 19S is well known (Kabat, 1943; Talmage, Freter and Taliaferro, 1956). Antibody activity in rabbit serum early in the response to antigen resides entirely in the γM fraction. After a short lag, antibody activity appears in the γG fraction, which has a sedimentation coefficient of 7S, and soon γM antibody is completely replaced by γG antibody (Bauer and Stavitsky, 1961). This sequential change is a general phenomenon, true for all antigens studied except possibly flagellin (Ada, Nossal, Pye and Abbot, 1963), and for all animals studied (Bauer and Stavitsky, 1961; Uhr, Finkelstein and Franklin, 1962; LoSpalluto, Miller, Dorward and Fink, 1962). γM antibody appears to be synthesized only while there is circulating antigen present. The appearance of antibody with subsequent immune clearance of antigen from the circulation leads to the cessation of γM antibody synthesis (Finkelstein and Uhr, 1964). Antibody activity in the γM fraction reappears transiently at the onset of an anamnestic response, but there is no heightened response as is true for γG antibody (Finkelstein and Uhr, 1964; G. F. Rosenquist and G. Campbell, personal communication).

There is ample evidence for the *in vitro* production of antibody and immunoglobulin by immunologically active lymphoid cells, but little is known about the synthesis of immunoglobulin by cells which are not thought to be involved in antibody production (Helmreich, Kern and Eisen, 1961; Helmreich, Kern and Eisen, 1962; Fleischmann, 1963). In 1940, Landsteiner and Parker cultured chick fibroblasts *in vitro* using nutrient medium which contained rabbit plasma and rabbit embryo extract but lacked chicken serum. The medium from these cultures was found to react by precipitation with a rabbit antiserum to chicken serum. The fibroblasts were kept in culture for as long as 7 months and subcultured many times to reduce the possibility of contamination of the media by chicken serum present in the cells at the time of explantation and to increase the probability of a pure fibroblast culture. The media continued to react with the rabbit antiserum. No further

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effort was made to identify the protein being synthesized by the cultured cells. There have been other reports in the literature of immunoglobulin production by chick muscle tissues cultured *in vitro* (Abdel-Samie, Broda, Kellner and Zischka, 1959; Abdel-Samie, Broda and Kellner, 1960). However, the methods used were inadequate to establish that the newly synthesized protein was immunoglobulin. A subsequent attempt by these workers to establish this identity failed (Budzynski, Broda, Kellner and Frummel, 1962).

This report presents immunological and physico-chemical evidence that various cell types from 16-day chick embryos and adult chickens, including cloned muscle fibroblasts, when grown in monolayer cultures can synthesize and release into the medium a 19S, γM-like molecule.

MATERIALS AND METHODS

Cell cultures

1. *Nutrient medium.* The basal nutrient medium was a modified Eagle's medium which has been previously described (Baluda and Goetz, 1961). Radioactive medium was made up by replacing the normal complement of serine with ^{14}C-labelled serine (specific activity 89–100 mc/mM) in the amount of 0·2 μc/ml. Immediately prior to use, chicken serum which had been heated at 56° for 30 minutes was added to give a final concentration of 5 per cent. The same pool of chicken serum was used for the entire study. Glutamine and Difco-Bacto-tryptose phosphate were also added to give a final concentration of 2 and 10 per cent respectively.

2. *Chick embryos and chickens.* All were of the White Leghorn breed (strain cross K-137) and were obtained from Kimber Farms Inc., Pomona, California. Embryonated eggs after 15 or 16 days of incubation at 38° and 4-month-old chickens served as donors of tissues.

3. *Cell cultures.* The bursa of Fabricius, yolk sac and thigh muscles were explanted, placed in separate dishes and minced with scalpels in Tris buffered (pH 7·4) isotonic salt solution (TBS). The cells were then dispersed with trypsin (0·25 per cent Bacto trypsin in TBS) at 37° until a homogeneous cell suspension was obtained, usually 15–30 minutes. After removing the trypsin and two washings with TBS by centrifugation, the cells were resuspended in nutrient medium. Approximately 5×10^6 cells in 5 ml of medium were plated per 60-mm diameter Falcon plastic culture dishes. The cell cultures were incubated at 38° in a humidified incubator and maintained at pH 7·2 by a continuous flow of mixture of CO_2 and air.

For peripheral blood cultures, adult chickens were bled by cardiac puncture into sterile syringes containing heparin. The blood was transferred to centrifuge tubes and the red cells were allowed to sediment for about 30 minutes. The overlying plasma which contained most of the white cells was then carefully removed. This white cell suspension was then used directly by plating about 5×10^6 cells in 5 ml of medium per culture dish. On other occasions the white cells were centrifuged and washed once in TBS before plating but this procedure was found to be unnecessary.

The nutrient medium was replaced at the time intervals noted, the interval never exceeding 4 days.

Immediately after explantation, fibroblasts from thigh muscles multiplied with a doubling time of about 1½ days and required subculturing. The proliferative activity of fibroblasts decreased markedly and eventually ceased after 4–7 weeks. In this study the cultures were used toward the end of proliferative activity. This insured the presence of a

relatively uniform cell type which could be studied over a long time interval without need for subculturing.

4. *Clones of fibroblasts*. Actively proliferating muscle fibroblasts that were in their fourth subculture, 12 days after explantation, were dispersed by trypsinization. After centrifugation, the cells were resuspended in 5 ml of TBS containing 10 per cent chicken serum. The clumps were allowed to sediment by gravity in a centrifuge tube for 15 minutes. The top half of the cell suspension was then transferred to another tube. This suspension consisted of single cells with less than 6 per cent of clumps of two cells. Ten dishes were then seeded with an average of ten or 100 cells in 5 ml of nutrient medium per dish.

After 16 days one macrocolony of cells appeared in one of the ten dishes seeded with only ten cells. The cells from this colony were dispersed with about 0·5 ml of trypsin and transferred to another dish. They reached a number of about 10^5, then ceased to multiply. This pure culture of fibroblasts was used in our study 34 days after plating and is referred to as CF4.

In each dish seeded with 100 cells four to ten macrocolonies developed. Thirteen well-isolated large colonies in ten dishes were dispersed with a drop of trypsin and each one was transferred to an individual dish. The cells in one of these picked colonies multiplied actively and had to be subcultured into two dishes 3 weeks later. These two cultures, referred to as CF_1, were used for our study 4 days later, when they contained about 10^6 cells each.

Antisera

Chicken γ-globulin was prepared from pooled normal chicken serum by ammonium sulphate precipitation at 45 per cent saturation. Approximately 40 mg of γ-globulin in 1 ml saline was mixed with an equal volume of Freund's adjuvant and injected into multiple sites in each of several rabbits. After 1 month the rabbits, which had high levels of precipitating antibody as determined by ring test and immunoelectrophoresis (IEP), were reinjected with similar doses of antigen without Freund's adjuvant. One week later, the animals were bled from the marginal ear vein. The sera collected were examined by IEP and pooled. The final pooled antiserum gave three distinct precipitin bands corresponding to γG, γA and γM by IEP. The antiserum was sterilized by filtration through Seitz filters, aliquoted into sterile tubes which were tightly stoppered and stored frozen until used. This is referred to in the text as anti γ-globulin serum.

Antiserum specific for chicken γM was produced, collected and stored as described above except that the ammonium sulphate precipitate was further fractionated by exclusion chromatography on Sephadex G-200 columns, and only the γM peak was used to immunize rabbits. One rabbit was found to be producing high precipitin levels of anti-γM, and virtually no antibodies cross-reactive with γG or γA. This antiserum was absorbed with material from the second peak of the Sephadex G-200 fractionation (primarily γG, γA and minor amounts of albumin) and subsequently gave only a single precipitin arc by IEP. This is referred to in the text as anti-γM serum.

Pea seedling globulin was prepared according to the method of Danielsson (1949) and sterilized by filtration. A portion was used to produce rabbit and chicken antisera as described above for γ-globulin. In the chicken, no Freund's was used and the secondary and tertiary injections came after 2 and 3 weeks, with final bleeding begun 1 week later. These antisera were pooled lots from sequential bleedings of several animals. They were sterilized by filtration, aliquoted into sterile tubes and stored frozen until used. The

remainder of the pea globulin was saved for use as antigen in subsequent heterologous, antigen–antibody precipitation absorptions.

Immune precipitation

1. *Heterologous immune precipitation absorptions.* Although antibody–antigen precipitation may be adequately specific, in complex systems including cell culture fluid some materials may add nonspecifically to the precipitate, and if these materials have been synthesized during the experiment they may have incorporated the radioactive label intended to indicate specific synthesis of the material precipitated. For example, complement would be nonspecifically incorporated in specific precipitates. An effective method of removing such materials from a system is to treat first with an antigen–antibody aggregate unrelated to the system under study, and to remove the nonspecific 'co-precipitins' that attach, by centrifugation. In the present study, pea seedling globulin (PSG) and antisera to this protein were used to remove nonspecific co-precipitants from the system. PSG was chosen because it is an easily obtainable, relatively pure protein which has no demonstrable cross-reactivity with chicken globulins.

Equivalence proportions for rabbit anti-PSG, chicken anti-PSG and the PSG solution were determined by the usual precipitin methods. These were done first in saline and then in culture medium. All precipitations were done under sterile conditions. To a constant volume of culture medium. PSG and rabbit anti-PSG were added in equivalence proportions. The solution was kept at 37° for 1 hour, by which time a flocculent precipitate was present, and then transferred to 4° for an additonal 20–24 hours. Two such precipitations were sufficient to remove all the nonspecific co-precipitating radioactivity. A subsequent absorption with PSG–chicken anti-PSG was also found to be unnecessary. When PSG was added to culture media alone, no precipitate formed.

After the second precipitation, the samples were centrifuged and the supernates transferred to sterile tubes for specific anti-γ-globulin precipitation. The precipitates were washed twice in cold 0·85 per cent NaCl and prepared for radioactive assay as described below.

2. *Specific precipitation.* Aliquots of twice-absorbed samples were treated with the rabbit anti-chicken γ-globulin at various concentrations to determine the amount of antiserum necessary to precipitate all immunoglobulins. The supernate of each test sample was examined by IEP for residual immunoglobulin. The lowest concentration of antiserum which precipitated all immunoglobulins from solution was used in all subsequent analyses. The precipitation schedule used was the same as that described for PSG–anti-PSG absorptions. The precipitates were washed in cold 0·85 per cent NaCl twice and assayed for radioactivity.

The absorbed antiserum specific for γM was used primarily for double diffusion in gel, or to demonstrate the amount of precipitable radioactivity in certain purified fractions of culture medium. These fractions were usually of limited volume. They were divided into equal portions and the specific antiserum added in increasing amounts to the different aliquots. The radioactivity in the precipitate was assayed and the test considered satisfactory when the radioactivity in the precipitate increased in the first two or three tubes and then reached a plateau.

Following the separation of the immune precipitate from its supernate, the latter was stored both at room temperature and in a refrigerator and frequently examined for any

additional precipitation. This was done to check the completeness of the previous immune precipitation and the maintenance of sterility.

Exclusion chromatography

Culture media were concentrated approximately twenty-fold by dialysis with powdered sucrose (McFarlane, 1964). The dialysis sack was retied to take up the volume change and the sample dialysed against 0·1 M Tris buffer, pH 8·0, in 1 M NaCl. One ml was applied to a Sephadex G-200 column ($60 \times 1{\cdot}5$ cm) at 4°. Using a Vanguard fraction collector, approximately 1 ml fractions were collected with a siphon measure. The fractions were monitored by absorbance at 280 mμ and 0·1 ml of each fraction were applied to filter paper disks for assay of radioactivity. Because siphon measures can show considerable variation in volume, the volume of each fraction was measured and cumulative volumes were used, rather than fraction numbers, in comparing different runs.

Ultracentrifugation

This was performed essentially as first described by Brakke (1953), using a linear sucrose gradient of 10–40 per cent on a Spinco Model L, SW 39 head, and run at 35,000 rev/min for 16 hours. Each tube contained 4·2 ml of sucrose solution and 0·5 ml of sample. At the conclusion of the run, fractions of five drops each were immediately collected after piercing the bottom of the tube with a pin.

Immunodiffusion

Ouchterlony (1948) double diffusion was performed in 1 per cent agar gel, made up in 0·075 M Veronal buffer, pH 8·6, and containing 0·85 per cent NaCl.

Immunoelectrophoresis

Immunoelectrophoresis was performed by the Scheidegger (1955) modification of the method of Grabar and Williams (1955) using the same agar gel described above but without NaCl. The samples were electrophoresed for 1·5 hours at a constant current of 8 mA per slide.

Starch block electrophoresis

Blocks of unhydrolysed starch ($12{\cdot}5 \times 45 \times 1$ cm) were used according to the method of Kunkel and Slater (1962). All samples were run at 4°, 400 V, for 20 hours using 0·1 M barbital buffer, pH 8·6.

The fractions were assayed for absorbance_{280} on a DU spectrophotometer. For radioactive counting, 3·0 ml aliquots of each fraction were mixed with TCA to a concentration of 5 per cent. The precipitates were separated by centrifugation and aspiration of the supernate. They were dissolved in 0·1 ml of 0·1 N NaOH and applied to paper disks.

Radioactive assay

The paper disk method described by Mans and Novelli (1961) for liquid scintillation counting was employed. Immune precipitates were dissolved in 0·1 ml 0·1 N NaOH and applied to Whatman 3MM paper disks (2·3 cm). 0·1 ml of saline wash of the tube was also added to the disk. Liquid samples were added to the disks in carefully measured volumes, usually 0·1 ml. The disks were dried in a 90° oven for 15 minutes and then batch-treated

with 5 per cent cold TCA, 5 per cent hot TCA, ethanol and ether in that sequence. Following the ether extraction, the disks were allowed to air dry and then were placed in glass counting vials to each of which was added 5 ml of scintillation fluid (0·3 per cent 2,5-diphenyloxazole, 0·03 per cent 1,4-bis-2-(4-methyl-5-phenyloxozolyl)benzene in toluene). Counting was done on a Packard Tri-Carb Scintillation spectrometer and all counts were corrected for background which never exceeded 150 counts per 10 minutes.

Radioactive counts of paper disks containing washed immune precipitates were found to be unaltered by the multiple extraction procedure outlined.

RESULTS

IMMUNE CO-PRECIPITATION

Experimental protocol called for the prior absorption of all media by a heterologous immune precipitate to remove labelled material which would tend to associate with an immune precipitate on a nonspecific basis.

Table 1

PSG–anti-PSG absorptions

	PSG–anti-PSG absorptions				Specific γ-globulin precipitation
	Rabbit antiserum			Chicken antiserum	
	1st	2nd	3rd	3rd	
Fibroblast (%)	24·1	1·3	<1	1·2	18·8
Yolk sac (%)	2·5	1·0	<1	2·0	16·2
Bursa of Fabricius (%)	7·6	4·2	<1	3·4	15·6

The percentage of TCA-precipitable protein co-precipitated with the PSG system and with rabbit anti-chicken γ-globulin. The first and second heterologous absorptions were done with 0·7 ml of medium. The third heterologous absorption and the specific precipitation were done with 0·2 ml of medium. All the samples were counted for 10 minutes and the counts were normalized to 0·1 ml.

Table 1 gives the results for culture media from three cell types absorbed by heterologous immune co-precipitation. The media shown had been added to the various dishes on the twelfth day of culture and removed on the sixteenth day. The data indicate that heterologous co-precipitation with rabbit antiserum and PSG is complete with two successive absorptions. A subsequent absorption with chicken antiserum and PSG removed additional nonspecific material from the media but in small amounts. Use of chicken antiserum in the heterologous absorptions would have introduced an additional variable into the system because an inconstant amount of carrier immunoglobulin would be added. This would have necessitated larger quantities of rabbit antiserum in the subsequent specific precipitation. The small amount of radioactivity brought down in the chicken anti-PSG precipitate was therefore not considered to justify the use of this additional step.

This test of the effectiveness of the heterologous co-precipitations was repeated several times. Similar results were obtained in each instance. Fibroblast media always gave much higher nonspecific co-precipitation with the heterologous material than did media from either of the other two cell types studied. In each case, however, the second co-precipitation was sufficient to complete the absorption.

Rabbit anti-chicken γ-globulin at equivalence proportion to the total chicken γ-globulin present was then added to the absorbed medium. The fraction of TCA precipitable protein which was specifically precipitated was determined. The results are shown in the last column of Table 1.

Nutrient medium containing [^{14}C]serine was incubated under the usual experimental conditions, but without cells, for periods as long as 2 weeks, and was analysed by the usual methods. Immune precipitates from these control media contained no measurable radioactivity associated with protein. In some experiments, non-radioactive serine in 1000-fold excess was added to aliquots of media at the time of harvest. There was no change in the amount of radioactivity present in the various precipitates. Storing the media at 4° instead of freezing them at −20° did not affect the results.

Experiments similar to those described above were carried out on cellular sap obtained from washed cells, suspended in buffered saline, which had been subjected to three cycles of freezing and thawing. The proportions of heterologously co-precipitated and specifically precipitated radiolabelled proteins were identical to those obtained with the proteins released by the cells in the surrounding medium.

The fractions of newly synthesized protein that could be specifically precipitated were determined at various time intervals after the cells had been placed in culture. Table 2 shows that the relative amounts of radioactivity incorporated into the immunoglobulin-precipitate rose slightly following explantation and reached a plateau by the second week.

TABLE 2

FRACTIONS OF SPECIFIC PRECIPITATE IN MEDIA AFTER VARIOUS TIME INTERVALS IN CULTURE

Sample	Days after explantation	TCA-precipitable counts per 10 minutes per 0·1 ml	Counts per 10 minutes in specific precipitate per 0·1 ml media	Total protein incorporated radio-activity in specific precipitate (%)
Fibroblast	4	11750	1347	11·5
	16	1802	355	19·7
	32	8516	1738	20·4
Yolk sac	4	6338	390	6·2
	16	3774	641	17·0
	32	6750	888	13·2
Bursa of Fabricius	4	10188	1027	10·1
	16	10982	1804	16·4
	32	11642	1722	14·8

Fractions of specific precipitate in media after various time intervals in culture. The media were collected every 4 days starting on day 4 after explantation. Media were absorbed twice with the heterologous PSG system, then equivalence proportions of rabbit anti-chicken γ-globulin were added. A volume of 0·2 ml of medium was used for each precipitation and the counts were normalized to 0·1 ml.

It appears from the experiments described above that various chick embryonic cells in culture continuously synthesize and liberate into the medium, a protein species which has the antigenic characteristic of a chicken γ-globulin. Although the major cell type by morphological and growth characteristics was quite distinct for each type of culture, it was also obvious that more than one cell type was present in each culture. Even the fibroblast cultures which always appeared the most homogeneous and unlike yolk sac and bursa cultures proliferated rapidly at first, might have contained a contaminating lymphoid cell type which was responsible for the synthesis of the protein species under study. In order to rule this out and to ascertain that the results obtained were valid for a pure culture of

fibroblasts, two cultures (CF_1), presumably derived from a single fibroblast and containing about 10^6 cells each, were studied for their ability to synthesize the protein species that can be precipitated by rabbit anti-chicken γ-globulin. The results are given in Table 3. Under the experimental conditions described, fibroblasts synthesize a molecular species which is precipitated by a rabbit antiserum to chicken γ-globulin.

TABLE 3

PROTEIN-INCORPORATED RADIOACTIVITY IN HETEROLOGOUS PRECIPITATE AND SPECIFIC PRECIPITATE OF MEDIA DERIVED FROM TWO CULTURES OF CLONED FIBROBLASTS

Day*		Counts in total protein	Counts in heterologous precipitate	Percentage of counts in heterologous precipitate	Counts in specific γ-globulin precipitate	Percentage of counts in specific γ-globulin precipitate
4	A	6912	757	11·0	340	4·9
	B	6979	749	10·7	346	5·0
8	A	12151	1882	15·5	713	5·9
	B	11918	1743	14·6	613	5·1
12		11278	1140	10·1	636	5·6
16		10554	858	8·1	630	6·0
20		9100	1242	13·6	524	5·8
24		7762	912	11·7	520	6·7

Protein-incorporated radioactivity in heterologous precipitate and specific precipitate of media derived from two cultures of cloned fibroblasts at approximately 10^6 cells per dish. A and B represent repeats on aliquots of the same sample run in parallel. All counts given have been normalized to 0·1 ml of medium except for total protein which was actually determined per 0·1 ml of medium. Heterologous precipitation was done with 0·7 ml media, specific precipitation with 0·25 ml media. Counts are per 10 minutes.

*[^{14}C]Serine medium was added to the cultures at day 0, 53 days after explantation, and replaced every fourth day.

The percentages of protein-incorporated radioactivity in the heterologous and specific precipitates are lower than those obtained previously but are consistent with them. This experiment and subsequent ones were done several months after those described previously. In the interim, a minor change in the procedure of counting the TCA-precipitable material in the media increased the counting efficiency to approximately double what it had been, leaving the counting efficiency of the immune precipitates the same. This resulted in more accurate figures which are approximately half their former value.

For optimal conditions the cell types studied required the addition of serum to the basal medium. Without serum the cells became detached from the dish, and died rapidly. The same events occurred, but more slowly, when purified bovine serum albumin or ovalbumin was substituted for the serum. It was also not possible to replace serum by hydrocortisone. In order to detect the effect that allotypic antigens, present in the chicken serum

TABLE 4

HETEROLOGOUS AND SPECIFIC PRECIPITATES IN MEDIA DERIVED FROM CULTURES OF ADULT PERIPHERAL BLOOD CELLS

Day	Counts in total protein per 0·1 ml	Counts in heterologous precipitate	Percentage of counts in heterologous precipitate	Counts in specific γ-globulin precipitate	Percentage of counts in specific precipitate
4	7254	76	1·0	248	3·4
8	6474	67	1·0	496	7·7
12	10714	193	1·8	709	6·6

Media were derived from cultures of adult peripheral blood cells cultured in 5 per cent autologous serum. All counts are per 10 minutes. Heterologous precipitation was done with 0·7 ml media, and specific precipitation with 0·25 ml media. Both were normalized to 0·1 ml.

used, might play in this study, peripheral blood leucocytes from adult chickens were cultured in medium supplemented with 5 per cent autologous serum. [^{14}C]Serine medium was used from the time of explantation and was replaced every fourth day. Table 4 shows that the nonspecific co-precipitation is low when compared to the previous results. This may be due to the different origin of the cells and to the absence of fibroblasts. However, the fraction of radioactive counts incorporated into the specific precipitate is similar to that obtained with other cells.

IDENTIFICATION OF γM-GLOBULIN

After showing that various types of chick embryonic cells synthesize *in vitro* a protein which has the antigenic specificity of chicken γ-globulin, the physical properties of this molecular species remained to be characterized. Its molecular size was determined in the following experiments.

Media from various cell cultures were concentrated and run on Sephadex G-200 columns to determine how the protein-incorporated radioactivity was distributed according to molecular size. In each case the whole concentrate was run and in some cases material precipitated by 45 per cent ammonium sulphate was run in addition. The results were identical for all types of cell cultures used. In the case of the ammonium sulphate precipitate the chromatogram lacked the usual albumin peak. Fig. 1 shows that all the radioactivity was in the first or excluded peak of the gel. The peak obtained in several runs was studied by immunoelectrophoresis with appropriate antisera. Fractions taken from the apex and from the leading edge of the first peak showed only a γM precipitation arc. The radioactive peak accounted for approximately 40–45 per cent of the original protein-associated radioactivity added to the Sephadex column; most of the remainder was never recovered and was presumed to be absorbed to the gel.

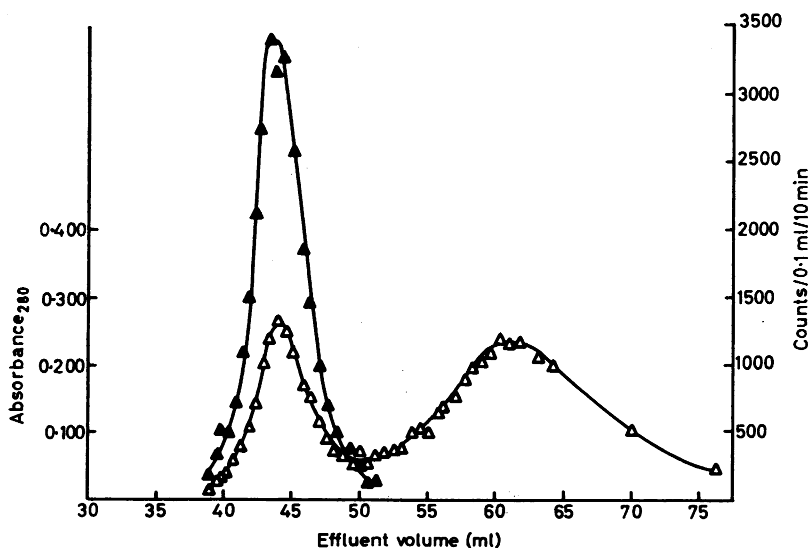

Fig. 1. Exclusion chromatography on Sephadex G-200 of concentrates of media derived from cloned fibroblast cultures. △, Absorbance$_{280}$; ▲, radioactivity.

The several fractions comprising the peak were pooled and dialysed against 0·85 per cent NaCl to remove the excess salt. This invariably resulted in the appearance of a fine precipitate which was insoluble in 0·85 per cent NaCl but soluble in 0·1 N NaOH, and in the loss of 40–50 per cent of the total protein-associated radioactivity initially present. Aliquots of the supernatant containing the other 50–60 per cent were treated with the heterologous PSG–anti-PSG system, with the specific anti-γ-globulin serum, with ammonium sulphate at 50 per cent saturation, or with 5 per cent TCA. The results are given in Table 5. The loss of heterologous co-precipitate, the relatively greater fraction of radioactivity in the specific immune precipitate (48 per cent vs. 6 per cent) and the much higher (81·6 per cent vs. 45 per cent) precipitability with half-saturated ammonium sulphate, reflect the loss of some non-γ-globulin contaminant.

TABLE 5

ANALYSIS OF EXCLUDED PEAK OF SEPHADEX G-200 RUN AFTER DIALYSIS AGAINST 0·85 PER CENT NaCl

	Percentage of total protein radioactivity in precipitate
Co-precipitation with PSG–anti PSG	0
Precipitation with anti γ-globulin antiserum	
0·1 ml	33·7
0·2 ml	48·7
0·3 ml	47·3
Precipitation with 45 per cent $(NH_4)_2SO_4$	81·6

Analysis of excluded peak of Sephadex G-200 run after dialysis against 0·85 per cent NaCl. Aliquot volumes of 0·3 ml of dialysed, pooled fractions from the excluded peak were treated with PSG–anti-PSG in equivalent proportions, with 0·1–0·3 ml volumes of rabbit anti-chicken γ-globulin serum, with 45 per cent $(NH_4)_2SO_4$, or with 5 per cent cold TCA.

To further characterize the physical nature of this globulin molecule, culture media from the various cell types, and from the cloned fibroblasts, were chromatographed on Sephadex G-200 and the fractions from the radioactive, excluded peaks were banded in a linear (10–40 per cent) sucrose density gradient. The collected fractions were analysed for radioactivity, and tested on Ouchterlony plates with rabbit anti-chicken γ-globulin and rabbit anti-chicken γM. The results are shown in Fig. 2. The major peak banded in the

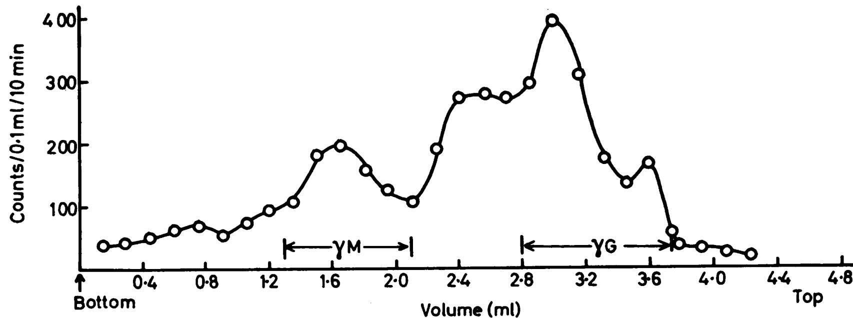

FIG. 2. Sucrose density ultracentrifugation of cloned fibroblast culture media (first or excluded peak of Sephadex G-200 chromatography). Linear gradient 10–40 per cent.

γG-globulin region and a second peak was present in the γM-globulin region. This relationship of the radioactive peaks to the sedimentation coefficients of 7S and 19S immunoglobulins was shown in a series of runs in which a drop of whole serum was added as a marker. Without this added serum, anti-chicken γ-globulin serum produced no precipitin arc by Ouchterlony analysis with the fractions of the 7S region, whereas arcs did develop in the 19S region.

The pattern shown in Fig. 2 was similar for all samples with one major exception. The shoulder on the left of the peak in the 7S region was seen only with fibroblast cultures, not with yolk sac or bursa of Fabricius cultures. This distinctive profile in the sucrose density gradient, and the fact that the PSG heterologous system always removed more non-specific precipitate from fibroblast medium than from the medium of other cultures, indicate that contaminating muscle fibroblasts were not responsible for γM-like globulin synthesis in the other cell cultures studied. This was further demonstrated by the synthesis of γM-like globulin by cultures of leukaemic cells and of normal peripheral blood leucocytes that did not contain fibroblasts.

The molecule which is present in the excluded Sephadex peak and bands in the γG region, is not immunoglobulin. It was of importance to demonstrate, beyond the limits of the Ouchterlony test, a clear antigenic difference between this contaminant and γM-globulin.

The sucrose density fractions in the γG and γM regions were treated with varying amounts of specific antiserum and absorbed by heterologous co-precipitation. The results are shown in Table 6. The radioactivity in the immune precipitates reached a plateau for the fractions in the γG region but not for those in the γM region. Unfortunately, there was insufficient material to carry this test further, but it is clear that the major immune precipitant resides in the 19S fraction.

TABLE 6

ANALYSIS OF FRACTIONS FROM THE SUCROSE GRADIENT ULTRACENTRIFUGATIONS BY SPECIFIC PRECIPITATION

Sample	Volume of rabbit antisera		Percentage of total protein counts in precipitate
	Anti γM	Anti γ-globulin	
A	0·05		15·5
	0·15		33·8
	0·25		57·0
		0·05	13·1
		0·15	28·7
		0·25	39·5
	Counts in heterologous precipitate		3·9
B	0·05		6·1
	0·15		10·5
	0·25		10·9
		0·05	4·8
		0·15	3·5
		0·25	3·9
	Counts in heterologous precipitate		1·2

Analysis of fractions from the sucrose gradient ultracentrifugations by specific precipitation. Sample A represents the fractions taken from the 19S region of the sucrose gradient. It contains γM only, by Ouchterlony analysis with or without added γM marker. Sample B represents the region of the gradient containing the γG marker. It failed to show a precipitin arc with Ouchterlony analysis when marker protein (whole chicken serum) was not added to the sample prior to the run. Aliquots of 0·2 ml of sample were used throughout.

In order to ascertain the identity of the γM-like globulin synthesized, fibroblast culture media were electrophoresed on starch blocks in the manner described by Wallenius, Trautman, Kunkel and Franklin (1957) for the analysis of human serum. The media were treated with ammonium sulphate at 45 per cent saturation. The precipitate was washed with a 45 per cent saturated solution of ammonium sulphate, dissolved in 0·85 per cent NaCl and dialysed against Tris–NaCl buffer. One-ml samples were fractionated on Sephadex G-200 columns. Fractions comprising the first peak of several column runs were pooled, concentrated by dialysis with powdered sucrose, and finally dialysed against 0·85 per cent NaCl to reduce the salt concentration. 0·5 ml fractions were layered on sucrose gradients and ultracentrifuged. The fractions comprising the 19S peak from three gradients were pooled and electrophoresed on starch blocks. The soluble material was eluted from the starch block segments and the $absorbance_{280}$ was measured. 3·0 ml aliquots of each fraction were treated with 5 per cent TCA. The precipitates were washed with 5 per cent TCA solution, dissolved in 0·1 N NaCl and placed on paper disks for counting.

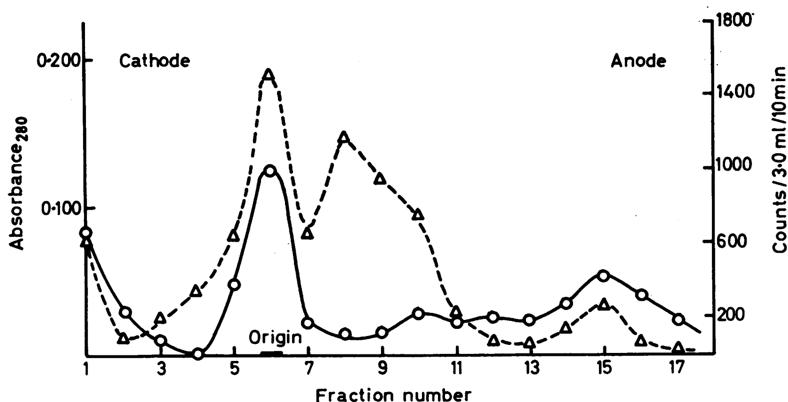

FIG. 3. Starch-block electrophoresis of pooled fractions taken from the 19S or γM-region of several sucrose gradient ultracentrifuge runs. Block was cut into 2·5 cm segments and extracted with three 2 ml washes of 0·85 per cent NaCl in Buchner funnel with a glass filter. The wash fluids were pooled and monitored by $absorbance_{280}$. A volume of 3 ml of each fraction was mixed with 1 ml 20 per cent TCA, and the precipitate was counted as usual. ○, $Absorbance_{280}$; △, radioactivity.

The results presented in Fig. 3 show a radioactive peak about the origin which is precisely at the position of γM-globulin. A second peak is seen in the β region, and a third in the α region; the latter may be the α-glycoprotein reported by Brown, Baker, Peterofsky and Kauffman (1954).

DISCUSSION

The data reported here provide strong evidence for the *de novo* synthesis of a protein with the antigenic and physico-chemical characteristics of γM-globulin by various chick embryonic cells, including cloned fibroblasts, in culture. Interpretation of the association of radioactivity with immunoglobulin, following the addition of nutrient medium containing a radioactively labelled amino acid to cell cultures, as a measure of the *de novo* synthesis of immunoglobulin, has been well documented (Keston and Katchen, 1956; Askonas, Humphrey and Porter, 1956; Stavitsky, 1958; Eagle, Piez, Fleischman and Oyama, 1959; Fleischman, 1963). The alternative possibility that this represents only

attachment of the labelled amino acid or peptides containing it to carrier immunoglobulins can be virtually eliminated. The labelling of protein is entirely dependent upon the presence of cells in the medium. The addition of unlabelled amino acid in large excess to media at the time of harvest, and subsequent mild treatment with 0·1 N NaOH, did not decrease the amount of radioactivity in the immune precipitate. The reaction is not dependent on the use of [^{14}C]serine; the substitution of [^{14}C]valine and subsequent analysis gave the same results.

In several experiments a commercially available, immunoglobulin-poor chicken serum (Hyland Laboratories, Glendale, California) was substituted for the normal chicken serum in the medium. These media were analysed as usual except for the necessity to add carrier protein in optimum amounts immediately prior to the immune co-precipitations. Exactly the same results were obtained as when normal chicken serum was used. This independence of the concentration of carrier immunoglobulin present during culture would not be expected if label was being attached to carrier immunoglobulin, and indicates that the label was being incorporated during active synthesis of immunoglobulin.

Finally, it is difficult to accept the possibility of a nonspecific external labelling which could involve γM and not γG. These two classes of immunoglobulins are similar in their constituent assembly. A nonspecific reaction would probably involve both, but there is no evidence that γG is ever involved with the radioactive label. Of course, the ultimate proof of *de novo* synthesis rests on peptide analysis of the γM-like fraction and finding a distribution of label among the serine-containing peptides.

The validity of the immunochemical data depends upon the specificity of the antisera for the antigens under study, and this rests mainly upon the purity of the antigens used for antiserum production. The antisera used in the immune precipitations reported here were developed in rabbits by injections of a mixture of chicken immunoglobulins. These antisera were tested on Ouchterlony plates with the antigen preparation and with the serum-containing media used in the experiment. In both instances the presence of antibody activity against only the three major classes of immunoglobulin was demonstrable. However, antisera with high titres of precipitating antibodies are usually derived, as here, from hyperimmunized animals that have had repeated large doses of antigen. Under these circumstances even minute contaminants may, after repeated injection, cause the appearance of antibody activity specific for the contaminants out of all proportion to the amounts present in the inoculi. It is prohibitively difficult to define completely the heterospecific activity of hyperimmune antisera. This possible complication must be considered whenever immune precipitation is used for analysis of complex protein mixtures, and must temper any interpretation of radioactivity incorporated into the immune precipitates.

Stronger evidence for the synthesis of γM-globulin was obtained by physico-chemical methods. Serum fractionation by exclusion chromatography on Sephadex G-200 allows good separation of γM and γG in the first and second protein peaks, respectively, with virtually no cross-contamination. Culture media from yolk sac, fibroblast clones, muscle fibroblasts, and bursa of Fabricius, of 16-day chick embryos, and from peripheral leucocytes of adult chickens gave identical results on Sephadex G-200. All the radioactivity appeared in the first peak excluding the participation of γG and of the majority of γA-globulins.

Sucrose gradient ultracentrifugation (Fig. 2) clearly demonstrated the presence of a protein with a sedimentation coefficient of 19S and of other materials of lesser sedimentation coefficients. Analysis of the various density gradient fractions by Ouchterlony

diffusion in agar gel and specific precipitation with rabbit anti-γM and anti-immunoglobulin serum showed that the 19S fraction contained the only radioactive precipitant (Table 6). Finally, the 19S fraction from the sucrose gradient ultracentrifugation was shown to have the electrophoretic mobility of γM-globulin by starch block electrophoresis as reported by Wallenius *et al.* (1957). This allows a clear separation of 19S γM-globulin and 19S serum α-glycoprotein.

There is also good quantitative agreement between the physico-chemical data and the immune precipitation data. The samples added to the Sephadex columns were first precipitated by 45 per cent ammonium sulphate which resulted in a recovery of 40–45 per cent of the original protein-associated radioactivity. The fractions comprising the first peak totalled approximately 50 per cent of the protein-associated radioactivity added to the columns. The recovery to this point was 20–22 per cent of the original protein radioactivity. These fractions were pooled and dialysed against 0·85 per cent NaCl. This resulted in the appearance of a precipitate with a concomitant loss of 40–50 per cent of the protein-associated radioactivity. These pools now contained 10–12 per cent of the original protein-associated radioactivity. As shown in Table 5, approximately 48 per cent of this radioactivity was precipitable by rabbit anti-immunoglobulin serum. This value is approximately 5–6 per cent of the original protein-associated radioactivity, and is in good agreement with the immune precipitation values shown in Table 3.

This quantitative agreement is not meaningful without the reasonable assumption that the protein lost in each of these steps is other than γM-globulin. Very little, if any, γM should have been lost in the ammonium sulphate precipitation and the Sephadex fractionation. The precipitate formed during dialysis was found to be insoluble in large quantities of 0·85 per cent NaCl and was presumed to be irreversibly denatured. This is more characteristic of serum α-glycoprotein as described by Brown *et al.* (1954) than the γM. There is also evidence from both starch block electrophoresis and ultracentrifugation that a labelled α-glycoprotein was present in the culture media studied.

There is little doubt that these media contained labelled proteins other than the γM-like protein with which this study was primarily concerned. Besides the α-glycoprotein mentioned there is labelled material which runs with the γM-like protein on Sephadex G-200, and has a sedimentation coefficient of 7S. This could be lipoprotein. There is a middle protein peak found on electrophoresis (Fig. 3) which is not identified and there are probably others. Very little can be said of any of these extraneous proteins because the antisera used had no demonstrated specificity for protein other than immunoglobulin and the arguments used for interpreting incorporation of label as synthesis and not external adsorption are not entirely applicable to protein species other than γM-immunoglobulin.

In spite of the weaknesses inherent in the methods used, such as the possible heterospecificities of the antisera and difficulties involved in the electrophoretic analysis of γM-globulin, the available evidence indicates that various chick embryonic cells, not generally associated with antibody production, synthesize a γM-like globulin.

The significance of this finding cannot be appraised fully since we do not know whether this γM-like globulin has antibody activity, or what role, if any, it may play in an immune response. Nevertheless, this work suggests that γ-globulin synthesis may be more widespread among cells than previously thought. Whether all the cells of an animal produce γM-globulin during its entire life span or whether they do so only during part of its embryological development remains to be determined. This observation should be

considered in the interpretation of studies on the synthesis of immunoglobulin by cells *in vitro* and on the mechanism of antibody production.

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