The Autonomous Production of Individual Serum Proteins by Tissue in Culture

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The production of individual serum proteins by individual kinds of tissue is difficult to prove or to disprove in experiments with intact animals or with surviving organs, since these contain a multitude of different kinds of tissue, often of overlapping activities and capable of mutual interaction. It is therefore preferable to investigate uniform tissue in isolation, i.e. in culture. Such tissue proliferates, in principle, indefinitely. Its biochemical potentialities presumably do not exceed those of the same kind of tissue *in vivo*, though they might be more restricted; thus the positive proof that a particular protein is synthesized by tissue in culture indicates that such synthesis may also proceed *in vivo*.

Intact animals or surviving organs are impregnated with fluids containing serum proteins, e.g. blood. It is therefore necessary when using them in experiments on protein synthesis to distinguish between pre-existing protein and newly synthesized protein. This can be done by the use of labelled amino acids, when only the newly formed protein will contain the label. Even in experiments with tissue cultivated in a medium originally free of serum proteins, however, the distinction between preformed and newly formed proteins is still needed in view of the protein contents of the cells themselves. For this reason, the use of isotopes is almost indispensable in this case as well. Moreover, the use of radioactive isotopes gives a method where the sensitivity of measurement is very high.

Three kinds of tissue were used for our experiments, namely mesenchyma tissue from chick embryos, epithelial-tumour HeLa tissue and a transformed human-liver-cell strain HLM. The tissue was incubated with nutrient medium containing [¹⁴C]amino acid, and subsequently the soluble proteins of the tissue and medium were separated and fractionated by selective precipitation with ethanol. Electrophoresis in starch gel, followed by staining, was carried out as a test for the purity of the fractions. Finally, the radio-

activity of the fractions separated with ethanol was measured; in one series (Table 3) the radioactivity of the eluates from the electrophoresis gels of such fractions was measured. The purity of the preparation of serum albumin has also been tested radio-immunochemically.

Various control experiments were needed to make sure that the results were not falsified by adsorption or other processes simulating genuine incorporation of amino acid. The most striking result of our work is the proof that both chick-mesenchyma tissue and HeLa-tumour tissue make serum albumin.

A preliminary report of some of our results has been given (Abdel-Samie, Broda, Kellner & Zischka, 1959).

EXPERIMENTAL

Materials

Radioactive amino acids. The makers of the radioactive amino acids (The Radiochemical Centre, Amersham, Bucks.) state that the specific activities of generally labelled L-[¹⁴C]tyrosine hydrochloride and DL-[1-¹⁴C]leucine were 54.3 and 41.6 $\mu\text{C}/\text{mg}$. respectively and that these amino acids were better than 99% radiochemically pure. They were used without further purification.

p-Fluorophenylalanine. This was supplied by L. Light and Co. Ltd.

Ethanol. A commercial grade of 95% ethanol was used without further purification, and the concentrations were determined by density measurement with a hydrometer at 25°.

Acetate buffers. Acetate buffers of known pH and ionic strength were prepared according to a nomogram (Boyd, 1945).

Serum-albumin antigens. The albumin for the immunochemical study was isolated by ethanolic fractionation and tested for its purity by electrophoresis in starch gel and staining (see below). Solutions (2%) of the albumin in 0.85% NaCl soln. were prepared, sterilized in the cold by filtration through a sintered-glass filter (Schott G5) under pressure, and kept ready for injection in 10 ml. vials in the refrigerator.

Rabbit antisera to serum albumin. Rabbit antisera against human-serum albumin (anti-HSA) and against chick-serum albumin (anti-CSA) were obtained by giving two intravenous injections of 5 ml. of 2% solution of

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electrophoretically pure serum albumin, with an interval of 1 week, followed by one intra-abdominal injection of 10 ml. 1 week later. The sera obtained by bleeding 10 days after the last injection were purified by absorbing the anti-HSA with chick serum and the anti-CSA with human serum. The titres were determined by adding constant amounts of the antiserum to different dilutions of the respective antigens and finding the lowest dilution of antigen at which it still gave a precipitate with the antiserum.

Methods

Calibration of antisera. A calibration of the antiserum was performed afresh each time with the radioactive fraction to be used. When increasing amounts of the antigen are added to a given quantity of antiserum, the amount of precipitate passes through a maximum (Heidelberger & Kendall, 1935, 1937; Pappenheimer, 1940). Therefore different serial dilutions of the antigen were added to a constant volume of antiserum. The optimum ratio for precipitation was found either by determining, at each point, the nitrogen in the precipitate, or by means of the ring test. The nitrogen was estimated by treating the precipitate in the Kjeldahl apparatus, distilling the ammonia, adding Nessler's reagent to the distillate and measuring spectrophotometrically (Beckman DU) the absorption at 420 m μ . For the purpose of the ring test, an antigen layer was introduced in a microtube on top of the antiserum, and the white ring between the layers was observed visually after 15 min. The ring appears fastest and shows maximum intensity at the ratio optimum for precipitation.

In treatment with anti-HSA, a good precipitate was obtained in any case because of the presence of a large amount of (non-radioactive) human-serum albumin in the nutrient medium. However, to obtain good precipitates with anti-CSA, it was necessary to add inactive chick-serum albumin as a carrier, as the nutrient medium contained only a small amount of material from chick, i.e. the embryo extract. Therefore the addition of 5–10 mg. of inactive chick-serum albumin to the whole amount of radioactive albumin obtained by ethanolic fractionation from one to two roller tubes was necessary before determining the optimum ratio for precipitation.

Tissue cultures. The mesenchyma tissue ('fibroblasts') was obtained from legs of chick embryos 11–12 days old and was grown as monolayers in roller tubes at 37°, as described by Kellner & Stockinger (1957) and Abdel-Tawab, Broda & Kellner (1959), in a nutrient medium

(1 ml./tube) consisting of 4% of chick-embryo extract, 40% of human ascites fluid and 56% of iso-osmotic buffered-salt solution (Gey, 1933).

Monolayers of HeLa cells were made in an analogous fashion from the HeLa tissue grown in these Laboratories for several years; HeLa tissue was originally derived from a human-cervix carcinoma (Gey, Coffman & Kubicek, 1952; Scherer, Syverton & Gey, 1953). The nutrient medium was the same as for fibroblasts except that the human ascites fluid was replaced by umbilical-cord serum.

HLM tissue is a transformed strain of human-liver cells from a 20-week male foetus (Sinclair & Leslie, 1959). It was grown in the same medium as HeLa tissue, but not as a monolayer.

Three days after the preparation of the fibroblast and HeLa-cell monolayers, either generally labelled L-[¹⁴C]-tyrosine (usually 0.5 μ C/tube) or DL-[1-¹⁴C]leucine (usually 0.85 μ C/tube) was added along with 1 ml. of fresh nutrient medium, and incubation was continued for a further 48 hr. The HLM-tissue cultures were incubated for 48 hr. with DL-[1-¹⁴C]leucine (usually 1.7 μ C/tube) in 1 ml. of fresh nutrient medium.

Separation of soluble proteins. After the end of incubation, the supernatant was decanted and the residual tissue was twice frozen in solid CO₂-ethanol and allowed to thaw. In this way the cell walls were ruptured. A small amount of quartz sand, 20 mg. of inactive carrier tyrosine or leucine and 2 ml. of 0.85% NaCl soln. were added, and the tissue was homogenized at room temperature within the roller tube by grinding for 5 min. with a rotating pestle. The cell debris and the sand were removed by centrifuging for 10 min. at 8000 rev./min., and the supernatant, together with the decanted nutrient medium, was subjected to ultrafiltration through a LSG 60 cellulose filter (Membranfilter-Gesellschaft, Göttingen) in a metal funnel under a pressure of N₂ of about 15 atmospheres (Manner, Broda & Kellner, 1957). The funnel was similar to the model MD 35-10 (Membranfilter-Gesellschaft). During the ultrafiltration, the metal was kept cool with ice cubes. The speed of filtration was about 2 ml./hr. The protein gel on top of the ultrafilter was washed five times with 5 ml. of 0.85% NaCl soln.

Experiments carried out with nutrient medium, in the absence of tissue, before and after incubation with the radioactive amino acid, showed that this procedure removed practically all radioactive compounds of low molecular weight from the protein (Table 1). High-molecular-weight compounds, such as soluble ribonucleic acid, will

Table 1. Removal of low-molecular-weight radioactive materials from protein by ultrafiltration and washing

Results in the absence of tissue; 0.5 μ C of tyrosine was added.

Sample	Not incubated		Incubated for 48 hr.	
	Activity (disintegrations/min.)	Fraction of added tyrosine (%)	Activity (disintegrations/min.)	Fraction of added tyrosine (%)
Filtrate	Not estimated	Not estimated	Not estimated	Not estimated
First wash (water)	120 000	12	100 000	10
Second wash (water)	3 900	0.39	4 000	0.4
Third wash (water)	570	0.057	760	0.076
Fourth wash (water)	190	0.019	220	0.022
Fifth wash (water)	100	0.010	95	0.0095
Protein remaining	10	0.001	17	0.0017

Table 2. *Conditions of fractionation of serum proteins*

(a) Human serum and plasma; (b) chick serum and plasma.

Fraction	Concn. of ethanol (%, v/v)		pH		Ionic strength	Temperature
	(a)	(b)	(a)	(b)		
I (Fibrinogen)	10	8	7.2	7.2	0.13	-2°
II (β - and γ -Globulin)	22	20	7.2	7.2	0.08	-5
III (α -Globulin)	40	36	5.8	5.8	0.09	-5
IV (Albumin)	40	38 ₁	4.3	4.8	0.11	-5

stay with the protein. As far as they may contain radio-carbon in the given conditions, discrimination between them and the protein is left to the subsequent stages.

Fractionation with ethanol. After the removal of the low-molecular-weight compounds, the proteins were redissolved in 0.85% NaCl soln., and submitted to fractionation by selective precipitation with ethanol-water mixtures (Cohn *et al.* 1946). This method was favoured as the ethanol and the low temperature prevent the growth of bacteria. In extensive tests to adapt the procedure to preparation on a small scale, it was found that optimum conditions of fractionation were obtained by adjusting concentration of ethanol, pH, ionic strength and temperature as shown in Table 2. In practice, conditions (b) of Table 2 were used in the experiments with chick tissue though the nutrient medium contained mostly proteins of human origin.

pH was measured with a Beckman pH meter or universal indicator paper (E. Merck). The cooling mixture, ethanol-solid CO₂, was kept in a large Dewar flask. The protein solution was contained in a large metal centrifuge tube, immersed in the flask, and closed by a rubber bung carrying a short tube for attachment to a filter pump, and a small funnel with a sintered-glass disk (G1), which was kept under the surface of the solution. Suction was applied to introduce the ethanol-water mixtures and the buffer solutions slowly into the centrifuge tube through the porous disk. The solution in the centrifuge tube was mixed by sucking air through the disk. After each step precipitate and supernatant were separated by centrifuging at -2° or -5°.

Starch-gel electrophoresis. Electrophoresis in starch gel (Smithies, 1955, 1959) was used in every case as a test for the purity of the fractions obtained by ethanolic fractionation. The electrophoresis was carried out on a portion for 5-6 hr. at a potential gradient of about 7-14 v/cm., corresponding to roughly 200-400 v in total, with a current of 20-25 ma. The temperature was about 8°. The regions of the proteins were located by running non-radioactive serum in parallel with the active sample, and staining with Amido-black in water-methanol-acetic acid (50:50:10, by vol.).

Electrophoresis was also used to separate the β - and γ -globulin, which are obtained together in fractionation with ethanol. For the measurement of the activities of the individual proteins, they were eluted with 0.85% NaCl soln. after destroying the starch gel by freezing, and the starch was removed by centrifuging.

Radioimmunological experiments. Serum albumin obtained by fractionation with ethanol and checked for purity by electrophoresis (plus 5-10 mg. of inactive chick-serum albumin when anti-CSA was used) was mixed with the quantity of calibrated antiserum required to give

optimum precipitation. The mixture was left standing for 2 hr. at room temperature and then kept in the refrigerator for 1-2 days; precipitate and supernatant were separated by centrifuging and the precipitate was washed successively with 2 ml. of 0.85% NaCl soln., 2 ml. of 5% trichloroacetic acid and 2 ml. of 50% ethanol. The supernatant was evaporated to dryness. Finally, the precipitate and the residue from the supernatant were subjected to combustion and the radioactivities were measured.

Reaction with ninhydrin. The proteins were hydrolysed by heating with 6N-HCl for 48 hr. at 120°, and the acid was removed by evaporation under vacuum. The sample (i.e. hydrolysed or unhydrolysed protein) was kept at 100° for 30 min., together with 50 mg. of inactive leucine (carrier), 300 mg. of ninhydrin and 20 ml. of citrate buffer (pH 2.5). During heating, a current of CO₂-free air was passed through the mixture, and the CO₂ evolved was absorbed in NaOH after removal of water vapour and volatile aldehydes with conc. H₂SO₄ and solid CO₂-ethanol traps. BaCO₃ was precipitated from the NaOH solution and its radioactivity measured. The yield in this system was found, by volumetric estimation of CO₂ evolved from non-radioactive leucine, to be nearly quantitative.

Measurement of radioactivity. For the measurement of radioactivity, the proteins were subjected to combustion to CO₂ by a wet method (Van Slyke & Folch, 1940). A known amount of inactive carrier (15 mg. of phenanthrene) was added to the sample before combustion to obtain a sufficient amount of CO₂. This was absorbed in NaOH and precipitated with Ba²⁺ ions. Finally, the CO₂ was liberated from the BaCO₃ and introduced into the gas Geiger counter (Broda & Rohringer, 1954), where the ¹⁴C β -particles are detected with 85% efficiency. The background of the counter, at various periods, was 40-50 min.⁻¹ The counting rates observed were converted into activities (disintegrations/min.) by the formula previously used (Suschny, Kellner, Broda, Figdor & Rücker, 1958).

The measurements of the radioactivities were often done with fairly small portions, often one-fifth or so, of the protein fractions, and the measured values had to be adjusted to the totals by multiplication. The reason was, in the experiments of Table 3, that only a small amount of protein could be introduced into the starch gel and, in those of Table 4, that a large part of each protein preparation was required to find the conditions of optimum precipitation. Therefore the relative statistical errors were high in samples of low activities, i.e. in the tails, though not in the main samples. For instance in column 2 of Table 3 (albumin) the measured activities (background deducted), with their standard deviations, were 155 $\pm$ 5, 10 $\pm$ 2.5, 16 $\pm$ 2.5, 2 $\pm$ 2, 5 $\pm$ 2.5, 5 $\pm$ 2.5 and 4 $\pm$ 2.5 min.⁻¹ respectively. In Table 4 (B, a; HeLa cells with tyrosine), the figures were 19 $\pm$ 3 for

precipitate with anti-CSA, 190 ± 5.5 for supernatant with anti-CSA, 206 ± 5.5 for precipitate with anti-HSA and 14 ± 3 for supernatant with anti-HSA. Zero activity has been entered in the tables when the activity actually measured was five disintegrations/min. above background or less, i.e. when the activity calculated to the whole of the original protein solution was less than about 75–150 disintegrations/min., depending on the portion measured.

RESULTS

Synthesis of serum proteins by tissue cultures. The activities of serum albumin and of α -, β - and γ -globulin were determined individually after incubation of chick-mesenchyma or HeLa tissue with radioactive L-tyrosine or DL-leucine. For this purpose, the fractions of serum albumin, of α -globulin and of $(\beta + \gamma)$ -globulin were isolated by ethanolic precipitation, the fractions subjected separately to electrophoresis and the radiocarbon in the regions corresponding to the different serum proteins was measured in every electrophoresis gel. The intermediate regions between the bands of the proteins were also measured. It is clear from Table 3 that ethanolic fractionation had worked well, and that the radiocarbon was contained, both in the chick-mesenchyma and HeLa tissue, in all four fractions (albumin, α -, β - and γ -globulin).

In parallel control experiments, nutrient medium for mesenchyma, without tissue, was incubated with radioactive tyrosine, and medium for HeLa and HLM tissue with leucine. Solutions of the total serum proteins were obtained as with the tissues. The total activities of these proteins were less than 0.3 % of those of the proteins produced in the presence of tissue and cannot be considered as significant.

Immunochemical identification of serum albumin. Immunochemical experiments were carried out with the serum-albumin fractions isolated by ethanolic precipitation and checked for purity by electrophoresis and staining. Measured portions of these fractions, obtained from all three kinds of tissue cultures incubated with radioactive amino acids, were treated with anti-CSA or with anti-HSA, and the radiocarbon in the precipitates and in the supernatants was determined in each (Table 4).

Table 3. Activities in the electrophoresis gels of the protein fractions from ethanolic precipitation

Region measured	Fractions		
	Albumin	α -Globulin (disintegrations/min.)	$(\beta + \gamma)$ - Globulin
(A) Chick mesenchyma was incubated for 48 hr. with 0.5 μ C of L-tyrosine			
Albumin	5 120	640	440
Intermediate	330	420	0
α -Globulin	530	3 300	0
Intermediate	0	330	0
β -Globulin	0	0	3 840
Intermediate	0	0	520
γ -Globulin	0	0	3 150
(B) HeLa cells were incubated for 48 hr. with 0.85 μ C of DL-leucine			
Albumin	15 300	940	1 330
Intermediate	2 000	620	920
α -Globulin	1 200	13 800	0
Intermediate	520	520	0
β -Globulin	0	490	13 580
Intermediate	0	0	1 480
γ -Globulin	0	0	12 200

Table 4. Immunochemical identification of serum albumin

Fraction tested	Treated with	Activity in	
		Precipitate (disintegrations/min.)	Supernatant
(A) Chick mesenchyma			
(a) 0.5 μ C of L-tyrosine (two incubations)			
Serum albumin	Anti-CSA	4 210, 4 370	305, 190
	Anti-HSA	495, 635	4 630, 3 660
α -Globulin	Anti-CSA	330	2 840
	Anti-HSA	300	2 400
(b) 0.85 μ C of DL-leucine			
Serum albumin	Anti-CSA	14 300	1 380
	Anti-HSA	1 185	13 120
(B) HeLa cells			
(a) 0.5 μ C of L-tyrosine			
Serum albumin	Anti-CSA	315	3 120
	Anti-HSA	3 400	225
(b) 0.85 μ C of DL-leucine (three incubations)			
Serum albumin	Anti-CSA	1 800, 1 490, 1 150	13 000, 14 400, 12 300
	Anti-HSA	14 100, 12 400, 11 100	1 190, 1 590, 800
(C) HLM cells (1.7 μ C of DL-leucine)			
Serum albumin	Anti-HSA	5 080	250

In blank experiments, run in parallel with those with tissue, the medium alone, in the absence of tissue, was incubated with radioactive amino acid under identical conditions, the soluble protein mixture was obtained by ultrafiltration and the albumin was precipitated from this mixture with anti-HSA. The activities of the precipitates were determined as usual, but were less than 2% of those of the albumin from comparable tissue experiments with chick-mesenchyma, HeLa or HLM tissue; they were considered as insignificant.

Table 4 shows that the radioactive protein of the serum-albumin fraction obtained with chick-mesenchyma tissue is precipitated specifically by anti-CSA but not by anti-HSA, whereas with the serum albumin from HeLa cells the opposite is true. The human-serum albumin from HLM-liver cells is also unambiguously identified as such.

Table 4 also includes some data obtained on treatment of α -globulin, isolated from chick mesenchyma by ethanolic precipitation and checked for purity by electrophoresis and staining, with anti-CSA and with anti-HSA. These data serve as additional controls both for the specificity of the antisera and for the sharpness of the separation between serum albumin and the globulins.

Action of ninhydrin on the radioactive protein. It has been pointed out above that the protein of the medium does not take up radioactivity. It remained to prove that the ^{14}C incorporated into the protein in the presence of tissue is present within the protein chain, that is, that the amino acid is bound within the protein in peptide linkage. This question was examined by the ninhydrin test (Winnick, 1950). Ninhydrin removes CO_2 from the carboxyl groups adjacent to free amino groups. Therefore radioactive CO_2 should be evolved from protein containing carboxyl-labelled amino acid on treatment with ninhydrin only after hydrolysis.

The ninhydrin test was applied (1) to a portion of the serum albumin from chick mesenchyma, isolated by ethanolic fractionation and tested for purity by electrophoresis and staining, and (2) to a portion of the total soluble protein from HeLa cells. Carboxyl-labelled DL-leucine ($0.85\ \mu\text{C}$) was added to the medium before incubation in each case.

With HeLa protein only 4.9% of the radiocarbon of the protein (13 800 disintegrations/min.) was found in the CO_2 set free with ninhydrin without hydrolysis, but 90.3% of the radiocarbon in the protein (12 400 disintegrations/min.) after hydrolysis. Similarly, with chick mesenchyma only 3.7% of the radiocarbon of the serum albumin (2420 disintegrations/min.) was found in the CO_2 without hydrolysis, but 84.4% of the radiocarbon (3290 disintegrations/min.) after hydrolysis. Evidently the amino acid was held within the protein chain in α -peptide linkage, and was released and

rendered capable of reacting with ninhydrin by hydrolysis.

Inhibition of protein synthesis by p-fluorophenyl-alanine. It was expected that new synthesis of proteins would be inhibited by p-fluorophenyl-alanine, an antagonist to the essential amino acid phenylalanine. If incorporation of leucine was unaffected, the results would point to a mechanism of incorporation of leucine other than protein synthesis afresh.

Monolayer cultures of HeLa tissue were used. The input was 600 000 cells/tube. Incubation with $0.85\ \mu\text{C}$ of DL-leucine lasted 48 hr. Some of the tubes were used for the determination of the radioactivity of the albumin after fractionation with ethanol and examination by electrophoresis. In eight to ten parallel tubes, the tissue was redispersed (converted into a cell suspension) with trypsin after 48 hr., and the cells were counted again.

In fact, heavy inhibition of incorporation into albumin was observed. However, the number of cells per tube at the end of incubation was also much reduced compared with the controls (Table 5). Fluorophenylalanine acts as a potent cell poison.

The numbers of cells during the 48 hr. of the experiment and the effects of the antagonist on their biosynthetic capacities are not known. Although in the circumstances no definite conclusion about the mechanism can be drawn, the parallel between the incorporation of leucine into serum albumin and the number of surviving cells suggests that the decrease in leucine incorporation is due to the drop in the formation of new serum albumin. Certainly there is no evidence that leucine is incorporated into preformed albumin. Such evidence would, of course, run counter to our general ideas about protein biosynthesis.

DISCUSSION

The method here described gives unambiguous information about the autonomous production of individual proteins by particular kinds of tissue. The new data show that chick-embryo-mesenchyma tissue produces chick-serum albumin, and both human-tumour HeLa tissue and transformed

Table 5. *Action of p-fluorophenylalanine on HeLa cells*

Concn. of fluorophenyl- alanine (mM)	Radioactivity of albumin (disintegrations/ min.)	Mean cell number after 48 hr. (% of control)
0	6480	100
0.01	5720	75.5
0.1	2960	33.8
1.0	650	9.2

human-liver HLM tissue produce human-serum albumin. Our conclusion that some extrahepatic tissues make serum albumin contradicts a widely held opinion. For instance it has been stated (Peters, 1957) that 'failure to find labelled carbon in the albumin from the particulate fractions of kidney, spleen, intestinal mucosa and heart after injection (of labelled amino acid) *in vivo* supports the wealth of previous evidence that the liver is the sole source of serum albumin (Miller, Bly, Watson & Bale, 1951; Popper & Schaffner, 1957; Dancis, Braverman & Lind, 1957)'. See also Miller, Bly & Bale, 1954.

We have not determined to what extent the amount of the serum proteins increases during incubation, i.e. whether there is a net production of these proteins. Eagle, Piez, Fleischman & Oyama (1959) have shown that strong incorporation of labelled amino acids into proteins can take place in tissue culture without net production of proteins. This 'dynamic' renewal of proteins can proceed at the rate of 1%/hr., whereas in conditions of maximum growth the amount of protein increases by 4%/hr. In every case, the incorporation takes place intracellularly. The mechanism of renewal is presumably identical with the mechanism for new synthesis.

Though it has now been shown that the machinery for making serum albumin exists within chick-embryo-mesenchyma and HeLa-tumour cells, it is not clear to what extent it is used *in vivo*. The serum-albumin production by the tissue *in vitro* can be estimated only crudely from the experiments here reported. We take Table 4 (B, b; HeLa cells with leucine) as a basis, and assume that the recovery of serum albumin after incubation with leucine was of the order of 50%, i.e. that the total activity of the serum albumin was about 25 000 disintegrations/min. Then it is found that about 1.32% of the initial amount of radioactive leucine (1.9×10^6 disintegrations/min.), i.e. 2.64% of the L-leucine, had been utilized to build up serum albumin.

For a calculation of the weight of this newly formed albumin, the effective specific activity of the free leucine is required. An approximate estimate is possible on the basis that the medium (which contains about one-half of buffered salt solution) has half the free leucine concentration of serum. This is about 25 $\mu\text{g./ml.}$ (Flaschenträger & Lehnartz, 1954). The concentration of the leucine will not change much during incubation, as essential amino acids are not formed anew, and, on the other hand, only a small proportion of the leucine was used. If further the crude assumption is made that the specific activity of the newly incorporated leucine is about half that of the free leucine in the medium (Loftfield & Harris, 1956;

Steiner & Anker, 1956; Askonas & Humphrey, 1958), the mass of the leucine incorporated into serum albumin works out as

$$12.5 \times 0.0264 \times 2 = 0.66 \mu\text{g.}$$

With a leucine content (by weight) of human-serum albumin of 11%, deduced from data given by Hughes (1954) and Peters, Logan & Sanford (1958), the quantity of albumin newly formed in 48 hr. is computed as 6 $\mu\text{g./roller tube.}$

This amount of new albumin is to be compared with the mass of the tissue. The mean dry weight of one HeLa cell is taken as $7.15 \times 10^{-4} \mu\text{g.}$ (Leslie, Fulton & Sinclair, 1957; see, however, Salzman, 1959), and each roller tube contained initially about 6×10^5 cells, i.e. the dry weight of tissue was 430 $\mu\text{g./tube.}$ If the change in the number of cells during incubation, which was not large, is neglected, the order of magnitude of the production of serum albumin in 48 hr. is obtained as 14 mg./g. of dry HeLa tissue, and in 1 hr. it was about 290 $\mu\text{g./g.}$

The protein content of one HeLa cell is given as $4.8 \times 10^{-4} \mu\text{g.}$ (Leslie *et al.* 1957), and 6×10^5 cells therefore contain 290 $\mu\text{g.}$ of protein. Thus the serum albumin produced in 48 hr., whether by net production or by renewal, corresponds to 2%, compared with the total protein content of the HeLa tissue. All the serum proteins taken together incorporate about 3.5 times as much leucine as the albumin alone (Table 3).

If similar calculations are carried out for chick-mesenchyma tissue (Table 4, A, b), and the weight of the cell is assumed to be two-thirds that of a HeLa cell (Manner *et al.* 1957), an hourly rate of albumin production of about 500 $\mu\text{g./g.}$ of dry tissue is reached. The order of magnitude agrees with that given for the production of serum albumin by sections of chicken liver, i.e. 120 $\mu\text{g./g.}$ of wet liver/hr. (Peters & Anfinsen, 1950). The production rate per unit weight is, however, larger than that calculated from the data by Sterling (1951) for whole man, or from the data by Jeffay, Winzler & Donnelly (1958) for rats.

Serum globulins are known to be formed, to varying extents, by several kinds of tissue. This applies also to the γ -globulins, and more particularly to the antibodies (Askonas & White, 1956; Askonas, Humphrey & Porter, 1956; Stavitsky, 1958; G. J. Thorbecke, personal communication). In the present investigation it has been shown that α -, β - and γ -globulin are formed by chick-mesenchyma tissue, by HeLa-tumour tissue and by HLM transformed-liver tissue. No qualitative differences between mesenchyma and HeLa tissue have been observed so far by us in respect to the production of any of the serum proteins.

Immunological work with the serum globulins is under way. In this work, the absolute rates of

production of different proteins are measured with better precision. The precision is improved by (1) adjusting the specific activity of the amino acid to a known value by adding known excess amounts of the non-radioactive amino acid before incubation, and by (2) determining the yield in the isolation of the protein fractions by isotope dilution after the addition of ^{131}I -labelled proteins.

SUMMARY

1. A method is described to establish the autonomous production of individual serum proteins by tissue in culture. The tissue is incubated with nutrient medium containing a ^{14}C -labelled amino acid (tyrosine or leucine), the soluble proteins are separated and purified by ultrafiltration, fractionation with ethanol and starch-gel electrophoresis, and the radioactivity of the individual proteins is measured.

2. The proteins of the nutrient medium contained no radiocarbon after incubation with the radioactive amino acid in the absence of tissue.

3. When tissue was incubated with medium and carboxyl-labelled leucine, and the protein after isolation was treated with ninhydrin, appreciable quantities of radioactive carbon dioxide were evolved only if the protein had been hydrolysed.

4. By these methods, the incorporation of radioactive tyrosine or leucine into serum albumin as well as into α -, β - and γ -globulin was demonstrated with chick-embryo-mesenchyma tissue, human-tumour HeLa tissue and human transformed-liver HLM tissue.

5. A crude estimate of the rates of production gave the result that 15 mg. of albumin was produced/g. of dry HeLa tissue in 48 hr., and 24 mg./g. in chick-mesenchyma tissue.

6. The identity of the labelled serum albumin was confirmed immunologically by treatment with antisera to chick- and human-serum albumin. In the experiments with chick mesenchyma, the bulk of the radiocarbon was found in the precipitate only after reaction with anti-chick serum albumin, whereas with HeLa and HLM tissue the opposite was true.

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