from a limited amount of isotope would probably be low, since the introduction of isotope is made at the beginning of a long series of chemical transformations and also because isotopic labelling of the solvent employed in the catalytic reduction is required. This investigator also considered introducing the isotope into ring *C* of an appropriate unsaturated C_{21} steroid. Accordingly, pregn-9-ene-3:20-dione was prepared from 11α-hydroxyprogesterone (Peterson & Murray, 1952) according to procedures described by Mancera, Ringold, Djerassi, Rosenkranz & Sondheimer (1953) and Rosenkranz, Mancera & Sondheimer (1954), but the isolated 9:11 double bond resisted hydrogenation with *cyclo*hexane as solvent and palladium–charcoal as catalyst. Further experimentation along these lines was halted in favour of work on preparing [16-^{3}H]progesterone as described above. Another approach, requiring isotope-exchange procedure, had also been considered, but this was not promising since Fukushima & Gallagher (1952) could thereby introduce very little stably bound deuterium into the progesterone molecule, although they obtained better results with certain other C_{21} steroids: the amounts of stably bound deuterium or tritium incorporated were small, but still useful for metabolism experiments. It might be pointed out that exchange procedures are inherently inefficient in the utilization of isotope (although the degree of incorporation may be very high indeed), which is not as great an economic consideration in the case of deuterium as it is with tritium.

The potential usefulness of hydrogen isotope as auxiliary tracers for carbon and their limitations must be considered in each particular case (an excellent discussion is given by Kamen, 1951). In the author's experience (unpublished experiments) [16-^{3}H]progesterone has proved very useful in studying the intermediary metabolism of the hormone, not involving loss of the C-17 side chain; the isotope would probably be lost in that event.

SUMMARY

A comparatively simple and inexpensive route for the preparation of [16-^{3}H]progesterone of high specific activity (0·5 c/m-mole) is described.

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The Synthesis of Serum Albumin and Tissue Proteins in Slices of Rat Liver and Liver Tumour

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There have been many attempts in the past to compare the way in which proteins are synthesized in normal and tumour tissue. However, for the most part the comparison has been made between the synthesis of the complex mixtures of proteins contained in the two types of tissue. Zamecnik (1952) has pointed out in his review how limited is the value of such comparisons. If the biosynthesis of proteins in tumour tissue does in fact differ from that in normal tissue, then such differences are more likely to be brought to light by comparing the synthesis of a particular protein in the two types of

tissue rather than by comparing the synthesis of mixtures of proteins. The present paper describes a system in which such a comparison might be made.

Peters & Anfinsen (1950*b*) demonstrated the synthesis of serum albumin in chick-liver slices. Later Miller, Bly, Watson & Bale (1951), using perfused rat liver, showed that the liver was the main, if not the only, source of serum albumin. The synthesis of albumin must, therefore, be considered a characteristic function of the liver cell. If liver cells retain this function during the transformation to malignancy, then it should be possible to study the synthesis of serum albumin both in liver and tumour.

The method of Peters & Anfinsen (1950*b*), in which the amount of albumin present in slices during the course of incubation is determined by means of a specific antiserum, has been adapted to the rat. This animal was chosen owing to the facility with which liver tumours can be induced by feeding with 4-dimethylaminoazobenzene. By including a radioactive amino acid in the medium in which the slices are incubated, not only is it possible to follow the changes in the amount of albumin present, but the rate of incorporation of amino acid into this protein may also be studied. Thus two criteria of protein synthesis are available.

The results of such experiments show that rat-liver slices synthesize serum albumin and that slices of liver tumour also possess this property. Experiments are described which indicate that the albumin is being synthesized by the malignant cells in the tumour slices and that the synthesis is not due to contamination of the slices with normal liver cells.

The incorporation of radioactive carbon from [^{14}C]glucose into the tissue proteins was studied with rat liver and liver-tumour slices by Zamecnik, Loftfield, Stephenson & Steele (1951). Later Campbell (1955) showed that the conversion of [^{14}C]glucose into radioactive protein was more efficient in liver-tumour slices than in liver slices. In view of these results the synthesis of serum albumin by liver and liver-tumour slices has also been studied in the presence of [^{14}C]glucose.

Preliminary accounts of some aspects of this work have already been reported (Campbell & Stone, 1955, 1956).

MATERIALS AND METHODS

Animals and production of tumours. Liver tumours were induced in male Wistar albino rats highly inbred at this Institute, by feeding on a diet containing 0·06% of 4-dimethylaminoazobenzene under the conditions previously described (Campbell, 1955).

When a group of ten animals was fed on a diet in which 3′-methyl-4-dimethylaminoazobenzene (Price, Harman, Miller & Miller, 1952) replaced 4-dimethylaminoazobenzene, fewer rats developed tumours, and in the affected livers the tumours were less discrete.

Histological examination. Pieces of liver and tumour were fixed in 0·9% NaCl containing 4% of formaldehyde, sectioned and stained with haematoxylin. To show the presence of glycogen, carmine stain was used. In several experiments, the tissues were examined by frozen section.

Radioactive compounds. Uniformly labelled D-[^{14}C]-glucose (100 μC≡1·64 mg.), [^{14}C]glycine (100 μC≡8·6 mg.) and L-[^{14}C]lysine (100 μC≡2·5 mg.) were supplied by the Radiochemical Centre, Amersham.

Preparation of rat-serum albumin

The method of Keltz & Mehl (1954), based on Cohn's method 10 (Cohn *et al.* 1946), could not be repeated successfully. Paper electrophoresis in veronal buffer, pH 8·6, showed that albumin was present in the early precipitates, and that the final albumin fraction was contaminated with an appreciable quantity of globulin. When the method of Ulrich, Li & Tarver (1954) was used, a greater yield of crude albumin was obtained, but it too contained a high proportion of globulin impurities.

The following method for the preparation of albumin was, therefore, adopted.

Serum was obtained from male rats by heart puncture. The serum was fractionated by the addition of an equal volume of saturated $(NH_4)_2SO_4$ solution to the serum diluted (1:1) with 0·9% NaCl. The precipitate was removed by filtration, and after dialysis the filtrate was freeze-dried. From 52 ml. of blood 886 mg. of crude albumin was obtained, which was further fractionated by zone electrophoresis.

Zone electrophoresis. The method first described by Flodin & Porath (1954), in which electrophoresis is carried out in columns which are subsequently eluted with buffer, was used. Details of the apparatus were as described by Porath (1954) except that the sintered-glass plate at the bottom of the column was replaced by a small pad of glass wool.

Partially acetylated cellulose, which was used to pack the column (54 cm. × 3 cm.), was prepared as follows. Absorbent cotton wool (300 g.), broken into small pieces, was refluxed for 20 hr. with 375 ml. of acetyl chloride and 4875 ml. of ethanol. The finely divided product was filtered off and washed first with ethanol and then with water. The larger particles were removed by flotation.

Electrophoresis was carried out in a borate–phosphate buffer (0·0115 M-$Na_2B_4O_7$–0·0155 M-NaH_2PO_4; I = 0·05. pH 8·6). After equilibration of the cellulose column with buffer, crude albumin (300 mg.) dissolved in 2 ml. of buffer was added to the column and washed 2–3 cm. into the cellulose. A current of 30 mA was applied for 42 hr. The protein was then eluted with the same buffer, at a rate of 10–15 ml./hr.; 2·5 ml. fractions were collected. The optical density of the fractions at 280 mμ was determined in a spectrophotometer with 0·5 cm. cells. The fractions containing the albumin peak were combined, dialysed and freeze-dried, yielding 213 mg. of albumin.

Antiserum against rat-serum albumin. Rabbits were injected with alum-precipitated albumin, as described by Campbell (1955). The amount of antibody in the rabbit serum was estimated by serial dilution to be at least 10 mg./ml. of serum.

Immunological examination. Immunological examinations of purified rat-serum albumin and of liver and tumour extracts, for antigenic heterogeneity, were carried out by

the combined electrophoresis and agar-gel diffusion technique of Grabar & Williams (1955). The antigen–antiserum reaction was also studied by the method of Oudin (1948).

Radioactive rat-serum albumin. A male rat (340 g.) was injected intraperitoneally with 50 μc (1·25 mg.) of L-[^{14}C]lysine in 0·5 ml. of 0·9% NaCl. After 6 hr. without food, but with water, the animal was injected intravenously with heparin and bled from the jugular vein, while the vena cava was perfused with saline. The blood–saline mixture was centrifuged and albumin obtained from the supernatant by fractionation as described above. The yield of albumin was 233 mg.

Incubation of slices and estimation of serum albumin

Incubation procedure. Slices of tissue were cut by hand and suspended in the bicarbonate medium described by Peters & Anfinsen (1950*b*). Outside slices of tissue were rejected and tumours, discrete and usually 0·5–2 cm. in diameter, were trimmed to remove unaffected liver before slicing.

To reduce the weight of albumin initially present, 4–5 g. of slices was washed in 100 ml. of medium by gentle agitation for 45 min. at room temperature. The supernatant was decanted, 50 ml. of fresh medium added, and washing resumed for a further 15 min. The slices were drained on filter paper, divided into approximately equal batches (0·5–2 g., depending on the experiment), and weighed and incubated in 125 ml. flasks containing 10 ml. of medium and the appropriate amount of radioactive compound at 37° in an atmosphere of $O_2 + CO_2$ (95:5). The flasks were shaken 100 times/min. during incubation. Kidney slices contained so little albumin that this tissue was not washed before incubation.

After incubation each flask was cooled in an ice bath and the contents were homogenized in a Potter-type all-glass homogenizer for 2 min. at 4500 rev./min. at room temperature. The pestle was tightly fitting. Care was taken to keep the procedure as uniform as possible. The tissue suspension was centrifuged at 15 000 *g* for 20 min. at 2°, the supernatant drawn off and diluted with an equal volume of 0·9% NaCl containing 1:10 000 Thiomersalate (sodium ethylmercurithiosalicylate). This solution, referred to throughout as a tissue extract, was stored at −20°.

Estimation of serum albumin in tissue extracts. The method of Peters & Anfinsen (1950*b*), as described in detail by Campbell (1955), was followed. Before the estimations, tissue extracts were spun at 15 000 *g* for 20 min. at 17°, to remove material precipitated by freezing and subsequent thawing. In some experiments it was necessary to modify the procedure as follows.

Separation of slices and medium before homogenizing. After incubating and cooling in an ice bath the mixture of slices and medium was centrifuged at 10 000 *g* at 0° for 8 min. The supernatant was drawn off and diluted with an equal volume of 0·9% NaCl containing 1:10000 Thiomersalate. The slices were homogenized in 5 ml. of ice-cold bicarbonate medium, and the homogenate was treated as described in the previous section.

Clearing extracts with chick-serum albumin–antiserum precipitates. Chick-serum albumin and the specific antiserum to this protein were prepared as described by Campbell (1955). Tissue extract (2 ml.) was incubated for 30 min. at 37° with 0·4 ml. of antiserum solution (10%, w/v) and 0·6 ml. of chick-serum albumin (0·1%). After storing overnight at 2°, the precipitate was spun off at 2° for 20 min. at 15 000 *g*, and the supernatant, now clear, used for estimation of albumin. The validity of this procedure is discussed later.

Measurement of radioactivity

Protein. Proteins were precipitated from tissue extracts by addition of an equal volume of 10% trichloroacetic acid. Non-protein material was removed as described by Zamecnik *et al.* (1951). The dried protein was plated on 0·28 cm.^{2} Perspex disks and counted at infinite thickness in a Geiger–Müller counter with a mica end-window.

Albumin. The radioactivity of the serum albumin in the tissue extracts was determined on the albumin–antiserum precipitates counted at infinite thinness on 2 cm.^{2} polythene disks as described in the Results section.

Glycogen and free-sugar estimations. In certain experiments the glycogen content of liver and tumour was estimated as follows. Weighed samples of liver and tumour slices (0·3–0·5 g.) (unwashed) were homogenized in 10 ml. of 5% trichloroacetic acid and glycogen was estimated according to the method of Kahan (1953), except that the modified anthrone reagent of Fairbairn (1953) was used. Free sugars were estimated in the solution in trichloroacetic acid (after treatment with $ZnSO_4$ and $Ba(OH)_2$) by the method of Hagedorn & Jensen (1923*a*, *b*).

RESULTS

Cytology of liver tumours

Rats which had been fed on the diet containing 4-dimethylaminoazobenzene showed considerable variation in the proportion of animals affected and in the gross appearance of the tumours. For this reason the tissue for any one experiment was generally taken from three or more animals, the slices being mixed before incubation. On several occasions liver slices from normal rats were studied, and this tissue will be referred to as 'normal liver', to distinguish it from the unaffected liver of tumour-bearing animals, which it is convenient to designate 'liver'.

Histological examination of the liver tumours showed that two types of malignant cells predominated. The first, hepatoma cells, are derived from liver cells, and the second, cholangioma cells, from the cells of the bile ducts. Some tumours contain a mixture of the two cell-types; others consist primarily of one type only. The only way of distinguishing hepatomas and cholangiomas is by microscopic examination. Since this could not be performed in every case, the malignant tissue used in most experiments probably contained both types of cells, and has therefore been referred to as 'tumour' tissue. In experiments in which the type of malignant cells predominating is specified, the tumours were classified by histological examination of frozen sections.

Histology of the tumours used for slicing showed that the amount of normal liver tissue present was only a small proportion of the whole. Nevertheless, it appeared desirable to devise a method of estimating the extent of the contamination of the tumour slices by normal liver tissue.

Edwards & White (1941) and Zamecnik *et al.* (1951) have shown that the glycogen content of liver tumour is much lower than that of liver. Histological examination of the tumours used in the present experiments confirmed this. Glycogen estimation seemed, therefore, to provide a suitable criterion for the presence of normal liver tissue. In three separate experiments the glycogen content (expressed as % wet wt. of tissue) in liver was 4·7, 4·7, 1·8, and in tumour 0·16, 0, 0. These results suggested that contamination of tumour with normal cells was very small but do not exclude the possibility that liver cells lying adjacent to tumour cells might have a very low content of glycogen. By selecting sections for histology which contained tumour and liver tissue it was possible to show that this was not so. It seemed unlikely, therefore, that the presence of liver cells in the tumour slices would complicate the interpretation of results obtained with such slices.

Rat-serum albumin

Fig. 1 shows the diagram obtained when the crude albumin fraction obtained by ammonium sulphate precipitation was subjected to electrophoresis on a small column of cellulose. The two smaller peaks are presumed to be α- and β-globulins. For comparison, Fig. 2 shows the diagram obtained when the albumin fraction obtained by alcohol fractionation according to the method of Ulrich *et al.* (1954) was subjected to electrophoresis under identical conditions. It will be seen that the preparation obtained with ammonium sulphate contains relatively less globulin than that obtained with ethanol, and since it was the simpler procedure it was adopted.

The crude albumin preparation was further fractionated on a cellulose column. The protein from the albumin peak was tested for homogeneity by electrophoresis in a Tiselius apparatus in a veronal buffer, pH 8·6, and in acetate buffer, pH 4·5. The results shown in Fig. 3 indicate that the albumin contained a small amount of globulin but there was no clear indication of a split in the albumin peak, such as was reported by Hoch-Ligeti, Hoch & Goodall (1949).

This albumin, which was used to prepare the antiserum, was further tested for heterogeneity by the agar-gel diffusion technique of Oudin (1948). This showed that it contained small quantities of a second component.

The combined electrophoretic and diffusion method of Grabar & Williams (1955) was also used to determine the composition of the albumin preparation. Thus in veronal buffer, pH 8·6, small

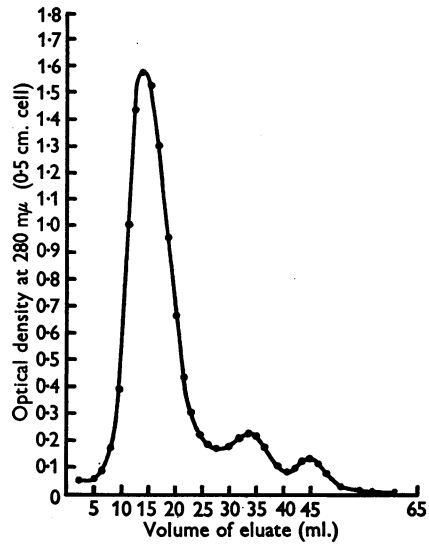

Fig. 1. Zone electrophoresis of rat-serum albumin obtained from whole serum after removal of the precipitate formed with 0·5-saturated $(NH_4)_2SO_4$. Protein (50 mg.) was added to the column (46 cm. × 1·5 cm.) packed with 'cellulose'. A current of 10 mA (500 v) was applied for 24 hr. Borate–phosphate buffer (see Methods section) was used.

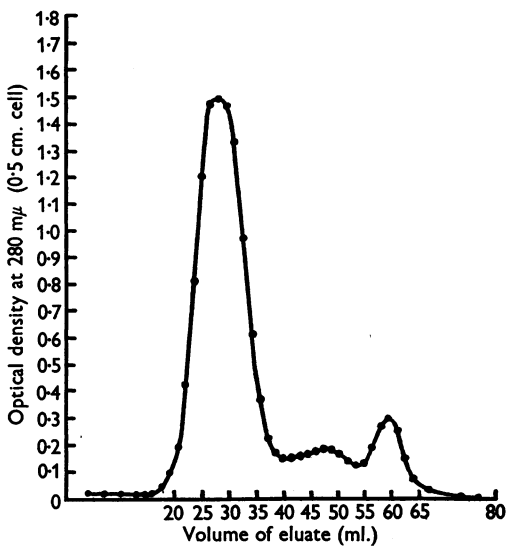

Fig. 2. Zone electrophoresis of rat-serum albumin obtained from whole serum by ethanol fractionation (see Methods section). The conditions were identical with Fig. 1.

quantities of a second component with a lower electrophoretic mobility than that of albumin were detected (see Fig. 4).

Specificity of antiserum

To test whether cross-reactions occurred in the tissue extracts between the antiserum and proteins other than albumin, liver and tumour extracts were examined by the immuno-electrophoresis technique. Since extracts which had been concentrated by freeze-drying failed to produce any precipitation with antiserum on the plates, presumably due to their low content of albumin, ammonium sulphate fractionation was used to concentrate the albumin in the extracts. The results, shown in Fig. 4, indicate that the antiserum reacted with only one component in the tissue extracts, which has an electrophoretic mobility similar to serum albumin. This is good evidence that the antiserum specifically precipitates the albumin in the tissue extracts and that the proteins precipitated in the extracts of liver and tumour are identical.

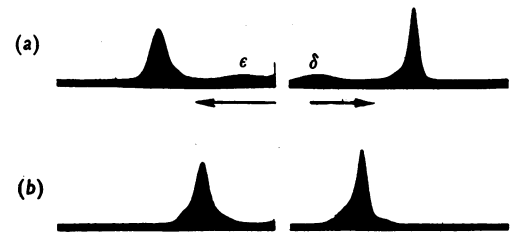

Fig. 3. Electrophoretic patterns of purified rat-serum albumin used for the preparation of antiserum. Each arrow indicates the position of the boundary at the start of the run and the direction of movement. Potential gradient, 4·5 v/cm. $I = 0{\cdot}1$, 1% protein. (*a*) Sodium veronal buffer, pH 8·59. Duration of run, 3 hr. 30 min. (*b*) Sodium acetate buffer, pH 4·52. Duration of run, 3 hr. 35 min.

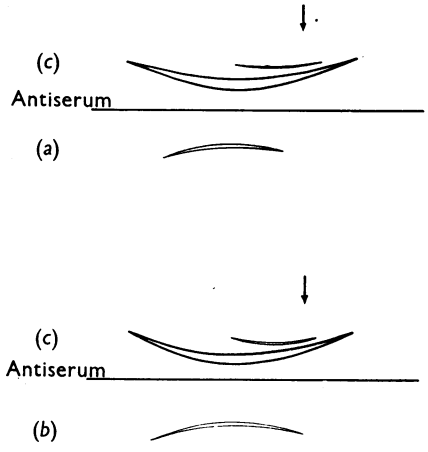

Fig. 4. Immuno-electrophoretic patterns of fractions of (*a*) liver and (*b*) tumour extracts compared with purified rat-serum albumin (*c*). Protein (2·5 mg. as 6% solution) was applied at starting position indicated by arrow, and current (40 mA, 150 v, for two agar plates arranged in parallel) applied for 6 hr., with sodium veronal buffer, pH 8·2, $I = 0{\cdot}05$. Antiserum (5%) was added to centre well and plates were stored for 14 days at 2° before photographing. (Diagrammatic representation.)

A similar experiment to that illustrated in Fig. 4 was carried out with fresh serum and purified albumin. Since the results were identical in each case it is concluded that the antiserum reacted with the same components in the fresh serum as in the purified albumin and did not cross-react with other proteins in the serum.

Estimation of rat-serum albumin in tissue extracts

Preparation of standard curve. When a standard solution of rat-serum albumin was assayed by the antiserum method the standard curve was very similar to that obtained with chick-serum albumin (see Campbell, 1955). Since small differences in incubation and centrifuging procedures were liable to affect the result slightly, a fresh standard curve was prepared for each assay. Duplicate estimations of albumin usually agreed to within ± 10% of the mean, although solutions very low in albumin could not always be assayed with such accuracy. On these occasions it was observed that the albumin–antiserum precipitate from the tube containing less albumin was disproportionately greater than the precipitate in the second tube.

Treatment of extracts with chick-serum albumin and antiserum. When attempts were made to estimate the amount of albumin in tissue extracts by the addition of antiserum, followed by estimation of the amount of precipitate, the results were rather variable. This seemed to be due in part to the presence of substances which were precipitated with the albumin–antiserum precipitate but which failed to dissolve in 0·1 M-sodium carbonate. Freezing and thawing the extracts did not consistently improve the results, nor did starving the animals overnight before taking the livers and tumours.

Askonas, Campbell, Humphrey & Work (1954) cleared whey-protein solutions by carrying out an ovalbumin–antiovalbumin precipitation in the extracts; the interfering substances were adsorbed on to the precipitate, which was removed. Chick-serum albumin and its specific antiserum were added to the tissue extracts in the present experiments. The effect of this treatment on the estimation of serum albumin in tissue extracts can be seen from Table 1 (Expts. VII and XI), in which the apparent level of albumin was appreciably lowered by the clearing process. This was most marked in liver-

tissue extracts, which before clearing were invariably more clouded than tumour extracts. Once the interfering material had been removed, albumin estimations were more readily reproducible. However, it is of interest to note that percentage change in albumin during incubation of slices was not greatly altered by clearing. For the latter reason it has been thought worth while to include among the results some estimations of albumin made on uncleared solutions, since these reflect changes in albumin content, if not absolute amounts present in different extracts.

The question arose whether clearing tissue extracts by a precipitation between chick-serum albumin and its specific antibody caused loss of rat-serum albumin, either by adsorption or by a cross-reaction with added antibody. The latter possibility was tested by incubating both standard solutions of rat-serum albumin and liver and tumour extracts with antiserum to chick-serum albumin. In no case was any trace of precipitate detected. The remaining possibility, that rat-serum albumin might be lost by adsorption, was tested by adding radioactive rat-serum albumin to an extract of normal liver and clearing this extract with chick albumin–antiserum. Since the chick albumin–antiserum precipitate was not radioactive it is clear that rat albumin had not been co-precipitated. In the experiments in which slices were incubated with radioactive amino acid and the extracts were cleared with chick albumin–antiserum the radioactivity of the rat albumin was not much affected by the process of clearing (Table 3). However, since the chick albumin–antiserum precipitate was significantly radioactive, the clearing of extracts is presumed to be due to adsorption.

Completeness of precipitation. To test for the presence of soluble albumin–antiserum complexes in the tissue extracts to which antiserum had been added, radioactive albumin (50 μg.) was incubated in the usual way with antiserum (3 mg.). The precipitate was spun off, and the supernatant dialysed, freeze-dried and 24 mg. of the product counted at infinite thickness on a 0·28 cm.^{2} disk. There was no detectable increase in counts above the background. This indicated that all the albumin had been precipitated by reaction with antiserum.

Estimation of specific radioactivity of albumin–antiserum precipitates

In spite of the fact that precipitation of albumin with antiserum increased the amount of protein available for counting by at least ten times, the amount of albumin likely to be present in tissue extracts was so low that counting at infinite thickness was impossible. Albumin was precipitated from tissue extracts under the conditions described for the estimation of albumin in such extracts. The

Table 1. *Synthesis of albumin in slices of liver from tumour-bearing rats and in slices of liver tumours*

Results are given in mg. of albumin/g. of wet tissue; percentage increase is shown in parentheses. Extracts were not cleared with chick albumin–antiserum precipitate except where stated.

Expt. no.	Liver			Tumour		
	0 hr.	2 hr.	4 hr.	0 hr.	2 hr.	4 hr.
I	1·98	2·82 (43)	2·77 (40)	0·74	1·12 (52)	1·35 (84)
III	1·05	1·87 (78)	2·63 (150)	0·55	0·74 (34)	1·19 (115)
VII	1·50	1·82 (21)	1·82 (21)	0·41	0·68 (66)	0·70 (71)
VII*	0·92	1·08 (18)	1·08 (18)	—	—	—
XI	1·29	2·22 (72)	2·34 (82)	0·49	0·69 (40)	0·88 (78)
XI*	0·70	1·09 (56)	1·35 (94)	0·43	0·62 (46)	0·76 (78)

* The extracts were cleared with chick albumin–antiserum precipitate before estimation of albumin.

Table 2. *Effect of amount of tissue on uptake of* ^{14}C *from a fixed glycine pool*

Different weights of liver and tumour slices were incubated with a constant amount of [^{14}C]glycine, and the radioactivity of albumin and TCA proteins was determined. In two separate experiments, A and B, slices were incubated for 2 hr. at 37° with 1·5 μc of [^{14}C]glycine (130 μg.). The results are expressed in counts/min./cm.^{2} at infinite thickness.

Wt. of tissue (g.)	TCA protein				Albumin			
	Expt. A		Expt. B		Expt. A		Expt. B	
	Liver	Tumour	Liver	Tumour	Liver	Tumour	Liver	Tumour
0·5	1327	2325	2813	4966	2088	452	3208	1720
1·0	1502	2209	2481	4716	2343	592	2250	1316
1·5	1414	1777	1839	3313	1972	383	1821	1022
2·0	1387	1262	1817	3159	2320	406	1656	726

protein was dissolved in 5N-NH_3 soln. and added in amounts between 100 and 700 μg. to disks (2 cm.^{2}). When dry these could be counted at infinite thinness, and the amount of protein on the disk determined afterwards by dissolving in 0·1M-sodium carbonate and measuring the optical density of the solution at 280 mμ.

Fig. 5 shows that amounts of protein up to 700 μg./2 cm.^{2} disk could be counted without requiring a correction for self-absorption. This was surprising, since 200 μg. of alanine was the maximum which could be counted at infinite thinness on a 2 cm.^{2} disk under the conditions described by Campbell (1955). The explanation appears to be that when a solution of amino acid is dried on a polythene disk the amino acid is deposited around the edge. Owing to the steep walls of the disk the counting efficiency is reduced. However, when a protein solution is dried on such a disk there is a more even distribution of protein over the whole disk and the counting efficiency is thereby increased. This is confirmed by the fact that the factor for conversion of the infinite thinness count/mg. of protein into infinite thickness on a 1 cm.^{2} disk is 4·8, whereas with an amino acid it is 7·6.

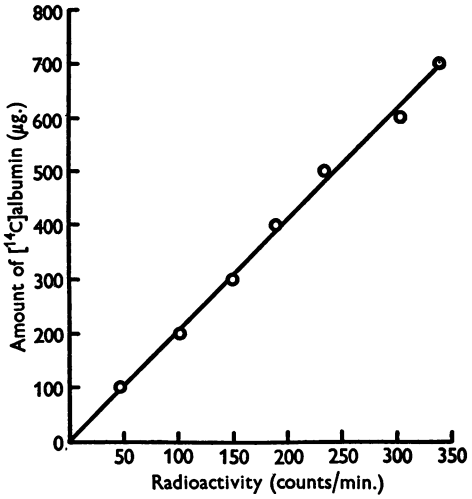

Fig. 5. Self-absorption curve of [^{14}C]-labelled albumin, for determination of specific radioactivity of albumin–antibody precipitates.

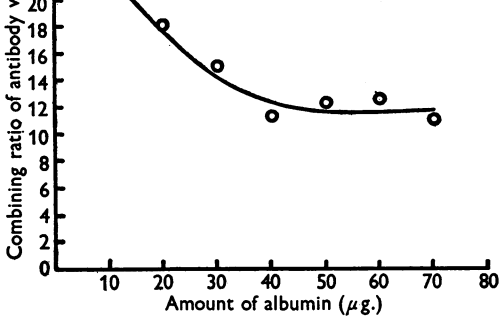

Fig. 6. Curve showing change in combining ratio of antibody with albumin. Combining ratios were calculated from the specific radioactivities of albumin–antibody precipitates obtained by treatment of various weights of serum albumin of known radioactivity with antiserum under standard conditions.

Combining ratios of rat-serum albumin with antibody. In order to determine whether the ratio of antibody to antigen varied when albumin was precipitated from solutions containing different concentrations of albumin, use was made of ^{14}C-labelled albumin. The specific activities of ^{14}C-labelled albumin–antibody complexes, prepared by adding different amounts of albumin (10–70 μg.) to a constant amount of antiserum (3 mg.) in solution, increased with increasing weight of added albumin until a constant figure was reached, when 50 μg. or more of albumin was used. Since the radioactivity of the albumin alone (475 counts/min./mg. at infinite thinness) was known, the weight of antibody combining with a fixed weight of albumin could be calculated. The results are shown in Fig. 6. Thus the ratio changed from 22·5 parts of antibody:1 part of albumin, at 10 μg. of added albumin, to 12:1, when 50 μg. of albumin was used.

Determination of specific radioactivity of albumin in tissue extracts. Owing to the variation in the combining ratio of antibody to albumin the radioactivity of the albumin in the different tissue extracts could not be directly derived from the specific radioactivity of the albumin–antibody complexes. Thus the concentration of albumin was first determined in a sample of the tissue extract. A further sample was then treated with antiserum and the specific activity of the albumin–antiserum precipitate was determined. Since the concentration of albumin in the extract was known, the combining ratio of albumin to antibody could be obtained from Fig. 6. Hence the specific radioactivity of the albumin in the extract could be calculated. In order to enable comparisons to be made the counts obtained at infinite thinness were converted to infinite thickness. Duplicate determinations of specific activity were within 10%.

Effect of amount of tissue on incorporation of [^{14}C]*glycine*

In order to determine the effect on the radioactivity of the tissue proteins of incubating different amounts of tissue slices with the same amount of radioactive amino acid, batches of tumour and liver

slices (0·5–2 g.) were incubated for 2 hr. in 10 ml. of medium containing the same amount of [^{14}C]glycine (1·5 μc, 130 μg.). Table 2 shows the results of two experiments in which the radioactivity of the proteins precipitated by trichloroacetic acid (TCA protein) and of serum albumin was determined. In Expt. A the radioactivity of both the TCA protein and the albumin remained relatively constant in liver, whereas in tumour the radioactivity of the TCA protein fell with increasing weight of tissue, and the albumins showed no definite trend. In Expt. B the radioactivity of both TCA protein and albumins in liver and tumour decreased with increasing weight of tissue.

Serum-albumin synthesis in liver and tumour slices

The results of estimations on the amount of albumin in liver and liver-tumour slices during incubation are shown in Table 1. In every case it will be seen that the amount of albumin increased during the incubation period, although there was considerable variation between the results of individual experiments. The results suggest that the albumin increase in the liver slices falls off more quickly than with tumour slices.

In Expt. VII, the effectiveness of washing liver slices before incubation was determined. Without washing, the level of albumin (in the uncleared extract) was 4·4 mg./g.; after 40 min. washing this had fallen to 2·37 mg./g., and at the end of 1 hr. the level was 1·5 mg./g.

The results of incorporation studies with [^{14}C]-glycine run parallel with the increases in albumin in tissue slices during incubation, for in every case the uptake of radioactivity by albumin increased with time. In some experiments incorporation of radioactivity into albumin was similar in the two tissues, whereas in others uptake by tumour albumin was lower than that by liver albumin. The results of a typical experiment are shown in Table 3. It will be observed that the radioactivity of the albumin is very much greater than that of the total soluble protein fraction of both liver and liver tumour.

Campbell (1955) showed that radioactivity from [^{14}C]glucose was more efficiently incorporated into the soluble protein of liver tumour than of liver when slices of these tissues were incubated with [^{14}C]glucose. These experiments have now been extended. Table 3 shows that not only is the soluble protein fraction from liver tumour very much more radioactive than a similar fraction from liver after incubation of slices with [^{14}C]glucose, but that the radioactivity of tumour albumin is also much greater than that of liver albumin.

Table 3. *Incorporation of radioactivity into albumin and TCA protein of liver and of tumour slices incubated with 2·5 μc of [^{14}C]glycine (Expt. XI, Table 1) or 4·5 μc of [^{14}C]glucose*

Radioactivity is expressed as counts/min./cm.^{2} at infinite thickness.

	Liver				Tumour			
Time of incubation (hr.)	... 2		4		2		4	
Precursor	Albumin	TCA protein	Albumin	TCA protein	Albumin	TCA protein	Albumin	TCA protein
[^{14}C]Glycine*	9072	2535	12 744	3613	6750	4512	11 502	6983
[^{14}C]Glycine†	8100	—	13 284	—	8208	—	11 556	—
[^{14}C]Glucose*	1404	63	1 620	111	7020	1128	9 126	1835

* Tissue extracts were not cleared with chick albumin–antiserum precipitate before precipitation of albumin.
† Extracts were cleared.

Table 4. *Synthesis of albumin and incorporation of radioactivity into albumin and soluble proteins of liver, hepatoma and cholangioma*

Slices were incubated with 2·5 μc of [^{14}C]glycine. Results are given in mg. of albumin/g. of wet tissue; percentage change is shown in parentheses. Tissue extracts were cleared before albumin was estimated or precipitated. Radioactivity is expressed as counts/min./cm.^{2} at infinite thickness.

Time of incubation (hr.)	... 0			4		
		Radioactivity			Radioactivity	
	Wt. of albumin (mg.)	Albumin	TCA protein	Wt. of albumin (mg.)	Albumin	TCA protein
Liver	0·82	0	17	1·11 (+35)	37 530	4524
Hepatoma	0·68	0	11	1·13 (+67)	18 252	4935
Cholangioma	0·96	0	15	0·90 (−6)	6 696	4274

Comparison of hepatoma and cholangioma slices

As explained in the Methods section, certain tumours were found by frozen section to consist predominantly of cells of bile-duct origin. The results of incubating cholangioma as well as liver and hepatoma slices with [^{14}C]glycine are shown in Table 4. An increase in serum albumin was found in both liver and hepatoma after 4 hr., but in cholangioma the level of albumin, initially higher than in either of the other tissues, fell slightly. These results, which suggested that little albumin synthesis was taking place in the cholangioma slices, were confirmed by the fact that the radioactivities of albumin in liver and hepatoma were six times and two to three times higher respectively than in cholangioma. On the other hand, the radioactivities of the TCA protein of all three tissues were approximately equal, showing that cholangioma was able to incorporate amino acids into the soluble proteins.

Comparison of liver and kidney slices from normal rats and of liver from tumour-bearing rats

Although there is no evidence that the kidney is able to synthesize albumin *in vivo*, kidney slices contained an appreciable quantity of serum albumin. Kidney therefore seemed to provide a suitable control to test the specificity of the observed synthesis of albumin in liver and tumour tissue. Kidney slices were incubated under the same conditions as had previously been used for liver and tumour. At the same time the opportunity was taken of studying the synthesis of albumin in normal liver slices. Young animals were used for these experiments, since preliminary experiments had shown that the rate of incorporation of radioactive amino acid into liver slices was greater in young animals than in old. As can be seen from Table 5, the results obtained with liver slices from normal animals are closely similar to those from tumour-bearing animals. With kidney slices the level of albumin fell slightly during the incubation and the incorporation of radioactivity into albumin was negligible. On the other hand, there was a considerable incorporation of radioactivity into the soluble-protein fraction, indicating that the tissue was capable of protein synthesis.

Albumin synthesis and secretion in liver and liver-tumour tissue slices

Fig. 7 shows the results of experiments in which the amount of albumin in tumour and liver slices and in the medium was determined during the course of incubation. It will be seen that slices of liver from normal rats and tumour-bearing rats, and slices of liver tumour, all gave similar results in that the total amount of albumin increased, the amount in the slices decreased and the amount in the medium increased. In Table 6 are seen the results of two similar experiments in which the slices were incubated with [^{14}C]glycine, and the incorporation of radioactivity into the albumin in the slices and medium was studied. The specific activity of the albumin in the medium was consistently lower than that in the slices both for liver

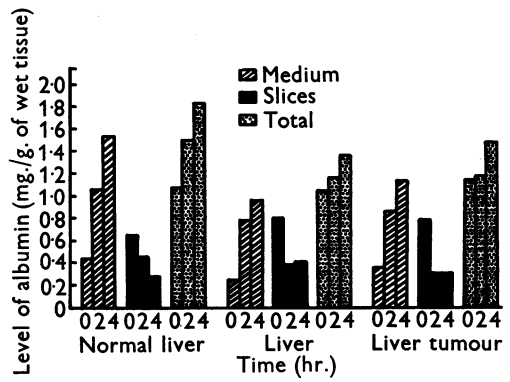

Fig. 7. Levels of albumin (mg./g. of wet tissue) in medium and in slices, and total albumin (medium + slices), during incubation of normal liver slices, slices of liver from tumour-bearing rats and rat-liver tumours. Samples at 0 hr. were stored in an ice-bath for 30 min. before homogenizing the tissue.

Table 5. *Synthesis of albumin and incorporation of radioactivity into albumin and soluble proteins of liver and kidney from normal rats.*

Slices were incubated with 2·5 μc of [^{14}C]glycine. Results are expressed as in Table 4.

Time of incubation (hr.)	Liver			Kidney		
	Amount of albumin (mg.)	Radioactivity		Amount of albumin (mg.)	Radioactivity	
		Albumin	TCA protein		Albumin	TCA protein
0	1·00	378	71	0·99	918	0
2	1·34 (+34)	7 398	1185	0·92 (−7)	756	596
4	1·93 (+93)	12 042	2599	0·88 (−11)	648	839

Table 6. *Incorporation of radioactivity into the serum albumin and soluble proteins precipitated by trichloroacetic acid of medium and slices during incubation of liver and liver-tumour slices with 2·5 μc of [^{14}C]glycine*

Radioactivity is expressed as counts/min./cm.^{2} at infinite thickness. Extracts were not cleared before albumin was precipitated.

Expt. no.	Time of incubation (hr.) ...		Liver 0	Liver 2	Liver 4	Tumour 0	Tumour 2	Tumour 4
V	Albumin	Medium	432	6 264	17 280	216	8 154	16 146
		Slices	108	8 262	19 980	216	12 690	17 064
VI	Albumin	Medium	1458	6 750	16 092	324	4 374	6 210
		Slices	—	15 174	31 536	216	7 452	12 366
	TCA protein	Medium	41	1 260	3 568	38	1 868	3 712
		Slices	—	1 192	1 551	13	2 462	3 071

and tumour, but there was no significant difference between the radioactivity of the TCA protein in the medium and slices.

DISCUSSION

Since the transformation of normal cells into malignant ones involves loss of morphological differentiation there has been a tendency to expect the characteristic biochemical functions of the normal cells to be lost in the process. That this is not invariably so has been shown in a number of tumours of endocrine origin. Thus certain metastases of adenocarcinomas of the thyroid in man retain many of the functions of normal thyroid tissue (Seidlin, Marinelli & Oshry, 1946) and adrenal cortical carcinomas can produce a mineralocorticoid effect (Foye & Feichtmeir, 1955). Hence it seemed possible that tumour cells derived from liver cells might retain the ability to synthesize serum albumin.

Peters & Anfinsen (1950*b*) showed that the amount of serum albumin in the supernatant from homogenates of chicken-liver slices plus medium increased during the course of incubation of the slices in a bicarbonate medium. Furthermore, when the slices were incubated in the presence of radioactive bicarbonate or glycine the albumin became radioactive. They therefore concluded that the slices were synthesizing serum albumin.

The results of the present experiments with rat-liver slices are similar to those of Peters & Anfinsen in that an increase in the amount of albumin in the slices plus medium during the course of incubation was invariably found. This was paralleled by the incorporation of radioactive amino acids into both the mixture of soluble tissue proteins precipitated by trichloroacetic acid and into the serum albumin precipitated by antiserum. The experiments with rat-kidney slices, in which there was a fall in the level of serum albumin during incubation, and a negligible incorporation of radioactive amino acid into the serum albumin, provide good evidence for the specificity of the synthesis observed in liver slices.

Khesin (1952) incubated rat-liver slices under similar conditions to those of Peters & Anfinsen and found that the amount of albumin in aqueous extracts of the slices and medium increased on incubation. However, when he treated homogenates of the slices by a procedure involving butanol he found there was no net increase in albumin in the slices plus medium during incubation. Khesin claims that the butanol procedure releases albumin from the cytoplasmic particles and that the increase in albumin observed with aqueous extracts is due to the release of preformed serum albumin. On the other hand, the levels of serum albumin found by Khesin in aqueous extracts of the slice homogenates were very much lower than those in the present experiments, and he did not study the incorporation of radioactive amino acids.

In the absence of exchange of amino acids with protein-bound amino acids the results of the present experiments must be interpreted as evidence that serum albumin has been synthesized by liver slices. The experiments with liver-tumour slices gave results which were qualitatively very similar to those obtained with liver slices, and it is therefore concluded that tumour slices also synthesize serum albumin. However, it was necessary to determine whether the synthesis in the tumour slices was due to the presence of normal liver cells within the tumour slices. The results of estimations of the amount of normal tissue in the slices on the basis of their content of glycogen, together with histological examination of the slices, suggested that this was not so. The failure of slices consisting mainly of cholangioma cells to synthesize albumin showed that the synthesis was confined to the hepatoma cells within the tumour slices.

Having shown that liver and hepatoma cells in slices behave in a qualitatively similar manner it is now necessary to turn to the quantitative aspects of the results. The result of four typical experiments in which the amount of albumin in liver and tumour

slices was estimated during the course of incubation are shown in Table 1. Although there are variations between the results of the different experiments, certain general conclusions can be drawn. Thus although the percentage increase in albumin in liver and tumour slices was not markedly different, the amount of albumin in tumour slices was consistently less than that in liver slices. For this reason the absolute amount of albumin synthesized in a given time by tumour slices was less than that by liver slices. If the average of the four experiments is taken, then liver slices synthesized 0·72 mg. of albumin/g. of tissue in 2 hr., and 0·94 mg. in 4 hr., whereas the corresponding figures for tumour were 0·26 and 0·48 mg. Thus the tumour slices synthesized between 30 and 50 % of the albumin synthesized by a similar weight of liver slices. It is of interest that with slices of chick liver Peters & Anfinsen (1950*b*) found an average increase of 0·12 mg. of chick-serum albumin/g. of tissue/hr., and Campbell (1955) obtained 0·43 mg. in 2 hr. and 0·90 mg. in 4 hr.

In this connexion the results of the determinations of rate of incorporation of radioactive amino acid are of interest. First, however, the effect on the radioactivity of the tissue proteins of incubating different amounts of tissue slices with the same amount of radioactive amino acid must be considered. The degree to which tissue proteins become labelled when slices of tissue are incubated with a radioactive amino acid must depend on the radioactivity of the amino acid present at the site of protein synthesis and the rate of protein synthesis. Thus in order to compare the rate of protein synthesis taking place in slices of different tissues it is necessary to know the relationship between the radioactivity of the amino acid precursor in the various tissues. If the amount of radioactive amino acid added to the medium in which the slices are incubated is large relative to the size of the pools of that amino acid in the slices, then it may be assumed that the radioactivity of the precursor amino acid in the various tissues does not differ widely, and a comparison of the rate of incorporation of that amino acid into the tissue protein will provide a comparison of the rate of synthesis of protein in the tissues. In this case there will be little variation in the degree of radioactivity of the proteins when different weights of tissue are incubated with the same amount of radioactive amino acid. This assumes that the concentration of amino acid is not such as to influence the process of incorporation (see Melchior & Tarver, 1947).

If, on the other hand, the amount of radioactive amino acid added to the medium is small compared with the size of the pools of amino acids in the tissue slices then it cannot be assumed that the radioactivity of the amino acid precursor in the different tissues is the same, and a study of rates of incorporation of radioactivity cannot be interpreted in terms of rates of protein synthesis. In this case the degree of radioactivity of the proteins in the tissue will fall as increasing amounts of tissue are incubated with a constant amount of radioactive amino acid.

The results of experiments designed to determine which of the above conditions applied to the present work are shown in Table 2. In Expt. A it will be seen that the radioactivity of the TCA protein from the liver remained fairly constant, whereas that of the tumour fell, with increasing amounts of tissue. The radioactivity of the albumin from both liver and tumour remained relatively constant in this experiment. In Expt. B, on the other hand, in which similar quantities of [^{14}C]glycine were used, the radioactivity of the TCA protein and albumin from liver and tumour fell with increasing amounts of tissue.

The results of Expt. A suggest that the pool size of glycine in the liver slices is smaller than that in tumour slices, whereas in Expt. B the evidence is that the two tissues are rather similar in this respect since the change in protein radioactivity with amount of tissue is similar in each case. Since the results of the two experiments are not identical it would appear that variations between different batches of slices may be great, and that unless the radioactivity of the precursor amino acid is determined in each case such experiments do not provide a satisfactory method of comparing the rate of protein synthesis in slices of different samples of tissue (for further discussion see Campbell & Halliday, 1957).

In view of the above considerations it is not possible to draw conclusions as to the relative rate of protein synthesis occurring in slices of liver and tumour from the rate of incorporation of radioactive amino acids into the protein of these tissues. However, since the slices from each type of tissue were well mixed before incubation, and the same weight of tissue was placed in each flask in any one experiment, the results obtained at 0, 2 and 4 hr. should be comparable.

When liver and tumour slices were incubated with [^{14}C]glycine the radioactivity of the albumin in the liver was greater than that in the tumour, whereas the reverse was true of the TCA proteins (Table 3). When [^{14}C]glucose replaced glycine the radioactivity of the albumin in the tumour was much greater than that in the liver and the difference between the TCA protein in the two tissues was even greater. Thus when the protein precursor was altered, the pattern of incorporation of radioactivity into the TCA proteins was reflected in a similar manner in the albumins. It therefore seems probable that albumin and the soluble tissue proteins are synthesized from the same pools of radioactive

amino acid. For this reason it is possible to compare the rate of albumin synthesis relative to the synthesis of TCA proteins in the two tissues.

If less albumin is synthesized in the tumour than in the liver slices, as has been suggested, then relative to the radioactivity of the TCA proteins the activity of the albumin should be less. That this is so can be seen from Table 3. After incubation for 4 hr. the TCA protein was 6983 counts in the tumour and 3613 counts in the liver; whereas the albumin in the tumour (11 502 counts) was less active than that in the liver (12 744 counts). If albumin had been synthesized at the same rate as the TCA protein in the two tissues the count of the tumour albumin would have been approximately double, which suggests that albumin was synthesized twice as fast in liver as in tumour. If the absolute amounts of albumin synthesized in the two tissues in this particular experiment are compared (see Table 1, Expt. XI) it will be seen that after 4 hr. the liver slices had synthesized 2·5 times as much albumin as had those from tumour. Thus the radioactivity measurements and the albumin estimates show good correlation, which suggests that the incorporation of radioactive amino acid takes place as a result of the synthesis of complete protein rather than by amino acid/protein-bound amino acid exchange. It may therefore be concluded that if the rate of synthesis of the soluble tissue proteins in liver and liver tumour is similar then serum albumin is synthesized at approximately half the rate in tumour slices that it is in liver slices.

It may be observed that the radioactivity of albumin in the liver slices was invariably much higher than that of the soluble tissue proteins. This is in agreement with the results of Peters & Anfinsen (1950*a*) and Campbell (1955) with chick-liver slices.

It remains to consider the results of the experiments in which the slices were separated from the medium after incubation. Peters (1953) found that when chick-liver slices were incubated with radioactive amino acid, and the radioactivity of the albumin in the medium plus slices was measured during the course of incubation, there was a delay in the incorporation of radioactivity into the albumin, whereas there was no delay in the incorporation of radioactivity into the liver protein as a whole. Peters interpreted these results as indicating that the conversion of amino acids into complete protein requires a measurable period of time and involves a 'polypeptide-like' intermediate.

When rat-liver and tumour slices were incubated with [^{14}C]glycine, the radioactivity of the albumin in the medium was consistently less than that in the slices (see Table 6). On the other hand, there was no such difference between the medium and slices in the activity of the total proteins precipitated by trichloroacetic acid. These results are consistent with those of Peters and show that the activity of the albumin in the medium lags behind that of the mixed tissue proteins. Such a lag could be explained if liver slices release albumin into the medium more rapidly than the other soluble tissue proteins, for relatively more unlabelled albumin would be released. This release of serum albumin seems to be a process requiring energy, for Khesin (1952) has shown that it is slower under anaerobic than under aerobic conditions. Thus albumin would appear to be secreted by the liver and tumour slices.

On the other hand, Peters found that when slices were incubated with radioactive amino acid and were then transferred to a non-radioactive medium the radioactivity of the albumin in the slices plus medium continued to increase, whereas that of the other tissue proteins slowly decreased. Such a result cannot be explained on the basis of a rapid release of albumin but would be expected if amino acids derived from the radioactive tissue proteins were utilized for the synthesis of albumin. The lag in the radioactivity of the albumin in the medium in the present experiments could also be explained on this basis. Further experiments are required to elucidate these points.

SUMMARY

1. The preparation of rat-serum albumin with ammonium sulphate fractionation and zone electrophoresis on columns of 'cellulose' is described. The homogeneity of the albumin was investigated by boundary electrophoresis and immuno-electrophoresis.

2. An antiserum, prepared by injection of rat-serum albumin into rabbits, was used to determine the amount of albumin in extracts of tissue slices which had been incubated in a bicarbonate buffer for periods up to 4 hr.

3. Tissue slices were incubated with [^{14}C]glycine and the incorporation of radioactivity into the soluble tissue proteins, precipitated with trichloroacetic acid, and into the albumin precipitated by antiserum, was studied.

4. A net synthesis of serum albumin occurred during the incubation of slices of liver from normal rats, and of slices of liver and liver tumour from rats bearing liver tumours induced by feeding on 4-dimethylaminoazobenzene. Radioactive amino acids were incorporated into both the soluble tissue proteins and the serum albumin of such slices.

5. Immuno-electrophoretic studies indicate that the albumin present in liver and liver-tumour slices is identical with serum albumin.

6. A net synthesis of serum albumin did not occur when rat-kidney slices were incubated, nor did the serum albumin contained in such slices become radioactive when the slices were incubated

with [^{14}C]glycine. However, the soluble tissue proteins did become radioactive under these conditions.

7. When slices of liver tumours consisting mainly of hepatoma cells were incubated with [^{14}C]glycine the serum albumin in the slices became radioactive and there was a net synthesis of albumin. However, when slices of liver tumours consisting mainly of cholangioma cells were incubated under the same conditions the incorporation of radioactivity into the serum-albumin fraction was very small and there was a net loss of serum albumin during the incubation. Such slices did, however, incorporate radioactivity into the soluble tissue proteins.

8. The incorporation of radioactivity from [^{14}C]glucose by slices of rat liver and liver tumour was also studied.

9. Radioactive rat-serum albumin was prepared from the serum of a rat which had received an injection of [^{14}C]lysine. This albumin was used to determine the combining ratio of serum albumin to antibody in precipitates of these proteins. The ratio was found to vary according to the concentration of albumin in the solution to which the antiserum was added.

10. The effect on the radioactivity of the soluble tissue proteins and serum-albumin fractions of incubating varying amounts of liver and liver-tumour slices with a constant amount of [^{14}C]-glycine was studied.

11. Slices of rat liver and liver tumour were incubated with [^{14}C]glycine and the radioactivity of the albumin and soluble tissue protein fractions was determined in the medium and slices independently. The results are interpreted as suggesting that serum albumin is 'secreted' both by liver slices and by liver-tumour slices.

12. From a comparison of the results obtained with liver slices and liver-tumour slices it is concluded that the hepatoma cells in liver tumours retain the ability of the liver cells to synthesize serum albumin. Determinations of the absolute amount of serum albumin synthesized by slices indicate that the tumour slices synthesize only 30–50 % as much albumin as do a similar weight of liver slices. Studies on the rate of incorporation of radioactive amino acid into the serum albumin in the tissues are in agreement with this observation.

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