these, but its function in the lens is entirely unknown and has scarcely been considered.

The lens of the rabbit has been examined more thoroughly than that of any other species for changes due to age or cataract formation. *meso*-Inositol, like glutathione (Pirie *et al.* 1955) and probably ascorbic acid (Kinsey, Jackson & Terry, 1945), but unlike ATP (Pirie *et al.* 1955), is at a lower concentration in the lens of the very young rabbit than in the adult rabbit (Table 1). Like glutathione (Pirie *et al.* 1953) and ATP (Pirie *et al.* 1955), it is mostly lost from a rabbit lens made completely cataractous by X-irradiation (Table 2), but limitations in the accuracy of estimation make it impossible to decide at what stage in the formation of cataract its concentration begins to decrease.

SUMMARY

1. Free *meso*inositol is found in the lens of several species of animal, ranging in concentration from about 50 mg./100 g. of lens in the rat to about 500 mg./100 g. of lens in the sheep and the human. This is higher than that recorded for any other animal tissue. It is present in the aqueous humour (rabbit, cattle) at a much lower concentration.

2. The concentration of *meso*inositol in the lens of the very young animal (cattle, rabbit) is lower than that in the adult animal of the same species.

3. There is no evidence of any combined form of *meso*inositol in the lens.

4. *meso*Inositol is virtually absent from the lens of a rabbit made completely opaque by means of X-rays, but limitations in the accuracy of its estimation do not permit conclusions as to the stage of cataract development at which the diminution begins.

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The Conversion of Glucose into Alanine and Glutamic Acid by Rat Liver, Liver Tumour and Kidney *in vivo*

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In studies on the metabolism of protein by tumour-bearing animals there often arises the problem whether a given protein has been synthesized by the tumour or by the normal tissues of the body. It seemed possible that this problem might be resolved by taking advantage of the differences in carbohydrate metabolism between tumour and normal tissues first described by Warburg (1930). He showed that, in general, tumour tissue has a higher rate of aerobic glycolysis than normal tissue. It might, therefore, be expected that the rate of turnover of oxoglutarate in tumour tissue relative to that of pyruvate would be lower in tumour than in normal tissue. As a result the rate of conversion

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of glucose into alanine and glutamic acid might differ in the two types of tissue. Thus if protein synthesis took place in the presence of [^{14}C]glucose the ratio of the radioactivity of glutamic acid to that of alanine in the protein of the two types of tissue might be different.

In this paper an attempt has been made to test this possibility by injecting [^{14}C]glucose into rats bearing liver tumours and determining the radioactivity of the alanine and glutamic acid in the liver and tumour protein after one hour. For the purposes of comparison kidney protein has also been studied.

To enable the conversion of glucose into amino acids in the tissues to be studied in greater detail, the incorporation into the tissue proteins of an essential amino acid which is relatively inert, [^{14}C]lysine, was also followed. Estimations were made of the radioactivity of the free alanine, glutamic acid and lysine in the tissues.

MATERIALS AND METHODS

Animals and production of liver tumours. Tumours were induced in rats by feeding them on a diet containing 0·06% of *p*-dimethylaminoazobenzene as described by Campbell (1955).

Radioactive substances. Uniformly labelled D[^{14}C]-glucose (100 μc$\equiv$1·29 mg.) and L[^{14}C]-lysine (100 μc$\equiv$2·5 mg.) were supplied by the Radiochemical Centre, Amersham.

Injection of [^{14}C]glucose and [^{14}C]lysine into rats. Three rats, weighing about 350 g. each, whose livers were infiltrated with tumour to varying degrees, were chosen after laparotomy. Thus rat 1 was badly infiltrated, rat 2 less so and in rat 3 the tumours were very small. At the time of injection, rats 1 and 2 had been on a normal diet for 2 months and rat 3 for 6 weeks, after the diet containing *p*-dimethylaminoazobenzene. All three rats were left for 1 week after laparotomy before injection. Each rat, under light ether anaesthesia, was given, by intravenous injection into the femoral vein, 1 ml. of a saline solution containing 20 μc of [^{14}C]glucose (260 μg.) and 5 μc of [^{14}C]lysine (125 μg.). After 60 min. the rats were anaesthetized with ether and each liver was perfused with 40 ml. of warm saline. The required tissues were cooled on ice.

Isolation of tissue proteins. The tumours were dissected free of liver tissue and liver tissue was similarly dissected free of tumour. Samples of liver, tumour and kidney tissue were then homogenized in a Potter-type glass homogenizer in cold saline. The tissue suspensions were centrifuged at 3000 *g* for 15 min. at 5° and the partially cleared supernatants were frozen overnight. The supernatants (*S*) were again centrifuged and filtered through Whatman no. 1 filter paper. The proteins were precipitated by addition of an equal volume of 10% (w/v) trichloroacetic acid and the precipitates (*P*) were heated to remove non-protein substances as described by Zamecnik, Loftfield, Stephenson & Steele (1951).

Isolation of free amino acids from tissues. The supernatant (*S*) obtained after the addition of trichloroacetic acid to the cleared tissue extracts (see above), together with the washings from the first treatment of the precipitated protein with 5% (w/v) trichloroacetic acid, was extracted three times with 3 vol. of ether to remove the acid. The amount of ninhydrin-positive substances present in the aqueous extracts was determined by the procedure of Cocking & Yemm (1954) (see also Campbell, 1955). The liver extract contained 24 mg. of amino acids calculated as alanine, liver tumour 48 mg. and kidney 17 mg.

The amino acids in the extracts were fractionated on columns of Dowex 1 (10% cross-linked, particle size 50 μ., 0·9 cm. diam., 30 cm. long) and Zeo-Karb 225 (W.R. 1·55, particle size 50 μ., 0·9 cm. diam., 100 cm. long; The Permutit Co. Ltd, London, W. 4) (Campbell, Jacobs, Work & Kressman, 1955) according to the method of Hirs, Moore & Stein (1954). With the Dowex 1 column 0·5M acetic acid was followed by 4M acetic acid and then N-HCl. With the Zeo-Karb column gradient elution was not used, approximately N-HCl (10% v/v AnalaR HCl) being followed by approximately 2·5N-HCl (25% v/v AnalaR HCl).

Since the capacity of the ion-exchange columns was limited, only one-half of the tumour extract was fractionated, whereas the whole of the kidney and liver extracts were used.

The fractions from these columns containing the required amino acids were concentrated and examined by paper chromatography, not only for ninhydrin-positive substances, but also for u.v. light-absorbing and -fluorescing substances. The concentrates containing glutamic acid from tumour and kidney were thus shown to be free from other ninhydrin-reacting substances. The concentrate containing glutamic acid from liver was contaminated by another ninhydrin-positive substance so that this fraction was further purified by paper chromatography with Whatman no. 3 paper and butanol–acetic acid–water (4:1:5, by vol.) as eluent (see Campbell, 1955). The fractions containing alanine were contaminated in every case with two other ninhydrin-positive substances and were, therefore, purified by paper chromatography as described for glutamic acid. The fractions containing lysine were always free from other ninhydrin-reacting substances, although the fraction from liver did contain a u.v. light-absorbing substance. For this reason some of the liver fraction was further purified on Whatman no. 3 paper.

Specific radioactivity of free amino acids (F). These determinations were carried out by counting at infinite thinness on 2 cm.^{2} polythene disks as described for alanine by Campbell (1955). The amount of amino acid on each disk was estimated by reaction with ninhydrin. It was found that when the specific activity of an amino acid was expressed as counts/min./mg. at infinite thinness the counts/min./cm.^{2} at infinite thickness could be calculated by multiplying the infinite thinness count by 7·6.

To ensure that the use of Whatman no. 3 paper for the final purification of the amino acids from the tissue extracts did not invalidate the determination of their specific radioactivity a control experiment was carried out with a standard solution of [^{14}C]alanine. The results showed that such a procedure did not significantly change the specific radioactivity of the amino acid.

Isolation of amino acids from proteins (P). Approximately 500 mg. of the tumour and liver proteins (*P*) was hydrolysed in a sealed tube with 10 ml. of 6N-HCl at 105–110° for 22 hr. After removal of excess acid the amino acids were isolated on columns of Dowex 1 (3 cm. diam., 30 cm. long) and

Zeo-Karb 225 (3 cm. diam., 100 cm. long) as described above. The effluent from the columns was analysed by paper chromatography and the fractions containing glutamic acid, alanine and lysine respectively were pooled. In every case, these fractions were uncontaminated by other ninhydrin-positive substances.

With kidney protein 60 mg. was hydrolysed with 2 ml. of 6 N-HCl as above. The amino acids from this hydrolysate were separated on small columns of Dowex 1 and Zeo-Karb 225 as described for the free amino acids.

The individual amino acids were isolated as follows: after removal of acetic acid glutamic acid was cryst. from aqueous ethanol. After removal of HCl alanine was converted into its dinitrophenyl (DNP) derivative according to the method described by Porter (1950). The reaction mixture consisted of 8 ml. of 50 % (v/v) aqueous ethanol, 100 mg. of anhydrous Na_2CO_3 and approximately 50 μl. of 1-fluoro-2:4-dinitrobenzene. The mixture was shaken for 2 hr. at room temp. The DNP-alanine was crystallized from a mixture of ether and light petroleum (b.p. 60–80°). Lysine was converted into its di-DNP derivative under the same conditions as for alanine but was crystallized from aqueous formic acid.

Specific radioactivity of amino acids from protein. All amino acids or their derivatives were counted at infinite thickness on either 0·28 cm.^{2} Perspex disks or 1 cm.^{2} polythene disks. The counts obtained for the amino acid derivatives were converted into the free amino acids for alanine and glutamic acid and to the monohydrochloride for lysine.

RESULTS

Radioactivity of amino acids from protein hydrolysates

The weights of the samples of the various tissues used, together with the weight of soluble protein precipitated by trichloroacetic acid obtained from each, are shown in Table 1. Table 2 gives the radioactivity of the amino acids isolated from the proteins precipitated by trichloroacetic acid. The coefficient of variation of these determinations was 3–7 %. For the determination of the average radioactivity of the amino acids from liver and tumour equal quantities of protein from each rat were hydrolysed together.

Radioactivity of the free amino acids from the tissues

It is, of course, very much more difficult to isolate samples of pure amino acids from tissue extracts than from protein hydrolysates. Thus, in many cases, fractionation of the tissue extracts on ion-exchange columns gave fractions containing more than one ninhydrin-positive substance. It was often necessary to purify the fractions further by partition chromatography on thick paper which had been extensively washed before use.

Although these methods of purification enabled fractions to be obtained which contained only one ninhydrin-positive substance it was thought that the fractions might contain significant amounts of substances which did not react with ninhydrin. It was for this reason that the chromatograms were scanned with ultraviolet light. In every case except that of the liver lysine the fractions were free of ultraviolet light-absorbing or -fluorescing substances. Further purification of the liver-lysine fraction by paper chromatography showed that the specific activity of the liver lysine was not significantly affected by the presence of the ultraviolet light-absorbing impurity. Small quantities of non-radioactive impurities in the tissue extracts would not be expected to affect the specific radioactivity

Table 1. *Amount of trichloroacetic acid-precipitable protein obtained from supernatants of homogenates of rat tissues*

Tissue wt. represents wet wt. of tissue. All weights in g.

	Liver		Liver tumour		Kidney	
Rat	Tissue	Protein	Tissue	Protein	Tissue	Protein
1	11·2	0·750	22·5	1·240	1·9	0·116
2	10·2	0·640	7·0	0·360	—	—
3	13·2	0·560	—	—	—	—

— Signifies values not determined.

Table 2. *Radioactivity of alanine and glutamic acid isolated from soluble tissue proteins 1 hr. after injection of rats with* [^{14}C]*glucose*

Radioactivity is given in counts/min./cm.^{2} at infinite thickness.

	Liver		Liver tumour		
	Rat 1	Average of rats 1–3	Rat 1	Average of rats 1 and 2	Kidney Rat 1
Alanine	158	132	232	186	46
Glutamic acid	56	50	78	52	41
Ratio alanine/glutamic acid	2·8	2·6	3·0	3·6	1·1

of the amino acids since the latter was determined at infinite thinness.

Since at least 500 μg. of 'pure' amino acid was always obtained it was possible to make several determinations of the specific radioactivity on each sample. The coefficient of variation between the various determinations was usually in the range of 6–9 %. However, where the counts were rather low, as with alanine and glutamic acid from kidney, the coefficient of variation rose to 15 %.

The results obtained from the determination of the specific activity of the free amino acids from the tissues of rat 1 are shown in Table 3 together with the activity of the protein-bound amino acids from the same tissues. It will be seen that in every tissue the F/P ratio for glutamic acid is higher than that for alanine. The difference between the F/P ratio of lysine for the three tissues is also significant.

Nature of the amino acids, peptides and related substances in the tissues

Although it was not the object of the present work to study differences between the free amino acids and peptides in the different tissues, some observations on these are of interest. Thus on Dowex 1 columns taurine was found in all the tissue extracts, but occurred in much higher concentration in those of liver than of tumour or kidney.

The behaviour of glutathione on columns of Dowex 1 was studied with an authentic sample. When the eluent on the column was changed after 95 ml. of effluent from 0·5 M to 4 M acetic acid, glutathione was eluted in a discrete band after a further 60–70 ml. Glutathione was thus easily detected in extracts of liver, whereas only traces were found in kidney extract and it was not detected at all in tumour extracts. This would appear to be a convenient way of isolating small quantities of glutathione. The low concentration of glutathione in liver tumour compared with liver is in agreement with the results of Zamecnik & Stephenson (1949), who estimated the concentration of glutathione in these tissues by an enzymic method.

After eluting the Dowex 1 columns with 4 M acetic acid they were washed with N-HCl and the concentrated effluent was examined by paper chromatography. No more than traces of ninhydrin-reacting substances were present in the tumour or kidney extracts, but the liver extract contained considerable amounts of such substances. When this fraction from liver was hydrolysed, there was a very marked increase in the number and amount of ninhydrin-reacting substances. This suggests the presence of conjugated amino acids in this fraction from liver.

The patterns of amino acids emerging from the columns of Zeo-Karb 225 were remarkably similar for the three tissue extracts. Asparagine was identified in all cases. Alanine was always contaminated by two unknown ninhydrin-reacting substances, the one with a peak just before the alanine peak and the other with a peak just after.

DISCUSSION

Following the demonstration by Warburg (1930) that the rate of aerobic glycolysis in tumours is higher than that usual for non-tumour tissues, it was thought possible that there is a difference in the pathway of oxidation of carbohydrate in the two types of tissue. In particular, the possibility that the tricarboxylic acid cycle might be relatively deficient in tumour as compared with normal tissue has been considered.

Table 3. *Radioactivity of amino acids in the supernatant fraction of homogenates of rat tissues* 1 *hr. after injection of* [^{14}C]*glucose and* [^{14}C]*lysine*

Radioactivity of the amino acids isolated from the protein precipitated with trichloroacetic acid is compared with that of the free amino acids in the extract. Radioactivities have been corrected to counts/min./cm.^{2} at infinite thickness. F/P ratios are expressed as $\pm$S.E.M. derived from the coefficient of variation of the radioactivity determinations of the protein and free amino acids.

Amino acid isolated	Protein amino acid (P)	Free amino acid (F)	Ratio F/P	$\frac{F/P \text{ lysine}}{F/P \text{ amino acid}}$
		Liver		
Alanine	158	2 519	16±1·6	0·38
Glutamic	58	1 275	22±2·6	0·28
Lysine monohydrochloride	980	5 978	6·1±0·54	1·0
		Liver tumour		
Alanine	232	6 396	28±2·9	0·34
Glutamic	78	3 572	46±2·6	0·21
Lysine monohydrochloride	1100	10 300	9·4±0·80	1·0
		Kidney		
Alanine	46	591	13±1·6	0·27
Glutamic	41	1 244	30±4·9	0·12
Lysine monohydrochloride	829	2 900	3·5±0·29	1·0

The very extensive studies which have been carried out on this subject have recently been reviewed by Weinhouse (1955). Although no qualitative differences in the metabolic pathways occurring in tumour and normal tissue have been found, marked differences of a quantitative nature have come to light. Thus Olson (1951) showed that slices of normal liver and liver tumour differed considerably in their metabolism of carbohydrate. It seemed possible that such differences might be reflected in the rate of conversion of glucose into alanine and glutamic acid by these tissues *in vivo*, which in turn might lead to a difference in the ratio of the radioactivity of alanine and glutamic acid when protein was isolated from the tissues after injection of [^{14}C]glucose.

The radioactivity of alanine and glutamic acid isolated from liver, liver tumour and kidney one hour after injection of [^{14}C]glucose is shown in Table 2. It will be seen that while the ratio of the radioactivity of alanine to that of glutamic acid from liver tumour is a little higher than the ratio from liver the differences are not very marked. However, the corresponding ratio for kidney is considerably lower than that of the other two tissues. Although this result shows that [^{14}C]glucose cannot be used to characterize protein synthesized by liver tumour as opposed to that synthesized by liver it provides little information regarding the rate of formation of glutamic acid and alanine from glucose in the tissues. It was for this reason that [^{14}C]lysine was injected with [^{14}C]glucose.

If an animal is injected with a radioactive amino acid, the rate at which this amino acid becomes labelled in the proteins of a given tissue depends on the radioactivity of the injected amino acid in the tissue and the rate of protein synthesis in that tissue. The permeability of the tissue to the injected amino acid and the amount of inactive amino acid with which the radioactive amino acid becomes equilibrated are likely to be the most important factors determining the radioactivity of the injected amino acid in a particular tissue. Thus the ratio of the radioactivity of a free amino acid in a tissue and that of the same acid in the tissue protein following the administration of a radioactive amino acid will be a measure of the rate at which that amino acid becomes incorporated into the tissue protein. In the absence of exchange between free amino acid and protein-bound amino acid the latter will be a measure of the rate of protein synthesis.

Ideally, it would be best to measure the ratio of the free amino acid and protein-bound amino acid radioactivity during the whole course of protein synthesis. In practice this presents difficulties. However, if the activity of the free amino acids is determined before complete equilibration between the amino acid pools in the tissues has taken place it is probable that the result will be indicative of the degree of dilution within the tissue of the injected radioactive amino acid. That complete equilibration of the free amino acids between the tissues had not taken place when the tissues were removed from the rat in the present experiments is indicated by the wide variation in the radioactivity of free lysine in the tissues (Table 3).

It is, of course, theoretically possible to inject so much radioactive amino acid that differences in the sizes of the pools of amino acids in the tissues are eliminated. Such a technique was attempted by Babson & Winnick (1954) by injecting massive doses of leucine into rats, but as Loftfield & Harris (1956) have shown, so much of the tissue amino acids are derived from the breakdown of tissue protein that it is doubtful whether this situation can be achieved in practice.

In the present experiments only 125 μg. of lysine was injected into each 350 g. rat. Since Sassenrath & Greenberg (1954) have shown that even the total plasma of such a rat would contain about 1 mg. of free lysine, it is unlikely that the amount injected in this case would significantly change the amount of free lysine available to the tissues.

From Table 3 it will be seen that the ratio of free tissue lysine to protein lysine one hour after injection of [^{14}C]lysine is in the descending order: kidney, liver, liver tumour. Since the free lysine had a very much higher radioactivity than the protein lysine, we may conclude that the rate of incorporation of amino acids is greatest in kidney, followed by liver and then liver tumour. This conclusion is only strictly valid if the incorporation of amino acid into a homogeneous protein is being studied. In the present experiments a mixture of soluble tissue proteins was used and no doubt these consist of components of differing amino acid composition having different turnover rates. Under these conditions the radioactivity of a particular amino acid at a given time after injection would depend on both turnover rates and the amino acid composition of the various components. Fortunately the concentration of lysine in proteins does not vary very greatly and it is possible that the soluble tissue proteins represent such a heterogeneous mixture that differences in turnover rates and amino acid composition would cancel out. Fractionation of such protein mixtures with ammonium sulphate, which was previously carried out (Campbell, 1955) on chick-liver slices after incubation with [^{14}C]glucose, showed no very striking differences in the radioactivity of the various fractions; nevertheless this possibility requires further investigation.

If proteins are synthesized from free amino acids and if the size of the pools of intermediates containing amino acids is very small, then it would be

expected that all the constituent amino acids would be incorporated into any given protein at the same rate. If, for the purpose of discussion, it is assumed that this is so and that there is no amino acid/protein-bound amino acid exchange, then the free amino acid/protein-bound amino acid ratio (F/P) would be expected to be the same for any one tissue no matter which amino acid was injected. The fact that such ratios for alanine and glutamic acid (Table 3) are in every case greater than those for lysine is a reflexion of the fact that glucose and not amino acid was injected. A delay in the time–activity curve for glutamic acid and alanine derived from glucose compared with that for lysine would, in any case, be expected.

An attempt has been made to express the rate of formation of alanine and glutamic acid from glucose by dividing the ratio of the free lysine/protein-bound lysine by the corresponding ratio for alanine and glutamic acid. The results are shown in Table 3. From these results we may conclude that the conversion of glucose into alanine occurs at approximately the same rate in liver and liver tumour, but is rather slower in kidney. Similarly, the rate of conversion of glucose into glutamic acid is rather lower in liver tumour than in liver, and is very much lower in kidney compared with liver. The results also show that the rate of formation of alanine from glucose in the three tissues is greater than the rate of formation of glutamic acid.

It may be concluded that if in fact the carbohydrate metabolism of liver tumour differs from liver *in vivo* these differences are not reflected as differences in the rate of conversion of glucose into amino acids in the two tissues. It should be emphasized that the liver tissue used in these experiments was taken from livers which were infiltrated with tumours. This tissue, which might be better described as precancerous, may differ in its metabolism from normal liver.

SUMMARY

1. Rats bearing liver tumours that had been induced by feeding *p*-dimethylaminoazobenzene were injected intravenously with a solution containing [^{14}C]glucose and [^{14}C]lysine. One hour after injection the animals were killed and soluble-protein fractions prepared from homogenates of the liver, liver tumour and kidney.

2. The soluble-protein fractions from the tissues were treated with trichloroacetic acid. The protein precipitate was hydrolysed and lysine, glutamic acid and alanine were isolated. The radioactivity of these amino acids was determined.

3. Free lysine, glutamic acid and alanine present in the supernatants from the trichloroacetic acid precipitates were isolated and their radioactivity was determined.

4. It is suggested that after the injection of a radioactive amino acid the ratio of the radioactivity of the free amino acid to that of the protein-bound amino acid in the tissue is a measure of the rate of synthesis of the proteins in the tissue. On this basis the rate of protein synthesis in the three tissues studied was, in descending order: kidney, liver, liver tumour.

5. From a comparison of the ratio of the radioactivity of the free amino acid to that of the protein-bound amino acid for glutamic acid and alanine compared with that of lysine it is concluded that (*a*) the rate of formation of alanine from glucose is similar for liver and liver tumour, but a little lower for kidney; (*b*) the rate of formation of glutamic acid from glucose in liver tumour is a little lower than that for liver, whereas for kidney it is much lower than for liver; (*c*) the rate of formation of alanine from glucose in the three tissues is greater than the rate of formation of glutamic acid from glucose.

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