Amino Acid Incorporation into Cell-Free Preparations of Normal and Neoplastic Lymphatic Tissues of the Rat

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Zamecnik & Keller (1954) showed that it was possible to obtain an energy-dependent incorporation of amino acids into protein when microsomes isolated from rat liver were incubated with soluble cytoplasm and radioactive amino acids. These experiments encouraged the hope that an understanding of the way in which proteins are synthesized by the cell might emerge from studies involving the incubation of subcellular particles. Ultimately it will, of course, be necessary to devise the means whereby the synthesis of specific proteins can be followed in such experiments, but before this is possible the requirements for the incorporation of amino acids into the protein of particulate fractions of many different tissues, each capable of synthesizing a different specific protein, must be elucidated. Some progress in this direction has been reported (e.g. Weiss, Acs & Lipmann, 1958).

It was decided to investigate the lymphatic tissues, because of their known ability to synthesize γ-globulin and antibody. In 1957, Wagle, Mehta & Johnson reported that nuclei-free suspensions isolated from rat spleen retained incorporating activity (see also Wagle, Mehta & Johnson, 1958). In preliminary experiments, preparations from spleen, lung and bone marrow therefore were investigated, but for various reasons most of the work to be reported in the present paper has involved the spleen. The incorporation of amino acids into preparations from transplanted rat lymphosarcoma has also been studied, for not only was it of interest to determine whether the tumour tissue differed from other lymphatic tissues in this respect, but also it seemed possible that a tumour tissue might offer interesting advantages as experimental material.

When a rat is injected with radioactive amino acids the most rapid incorporation of radioactivity in the liver is into the microsomal fraction of the cell (Keller, Zamecnik & Loftfield, 1954). Furthermore, when the subcellular fractions are isolated and incubated with a radioactive amino acid the energy-dependent incorporation of amino acid into the microsome fraction is greater than that occurring in the other cell fractions. On the other hand, if the extent of incorporation of radioactive amino acids into the subcellular fractions of the spleen is determined 20 min. after the injection of amino acid the mitochondrial fraction is the most active (Campbell, Greengard & Rendi, 1958). This suggested that this fraction of the spleen might also be the most active fraction under conditions *in vitro*. However, it will be shown that, as with the liver. the microsomal and not the mitochondrial fraction of the spleen is the more active.

In an attempt to determine whether there is any characteristic difference in the incorporation of amino acids into the microsomal fraction of the liver and spleen the extent of incorporation of various amino acids into this fraction of the two tissues has been compared. In fact, such differences were found and these have been used to study the role of the cell-sap fraction in determining the specificity of the incorporation of amino acids into the microsomes.

A preliminary report of some aspects of this work has already appeared (Rendi & Campbell, 1958).

MATERIALS AND METHODS

Chemicals. Adenosine triphosphate (dipotassium salt; ATP) and guanosine triphosphate acid (sodium salt; GTP) were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. and neutralized with KOH. The barium silver salt of phosphoenolpyruvic acid (PEP) was prepared by the method of Ohlmeyer (1951), and the dibarium salt of fructose diphosphoric acid (FDP) was obtained from C. H. Böhringer und Söhne G.m.b.H., Mannheim, Germany. A solution of the free acids was prepared shortly before use and was adjusted to pH 7·8. Glutathione was obtained from The Distillers Co. (Biochemicals) Ltd., Liverpool. PEP-kinase, obtained either from C. H. Böhringer und Söhne, or prepared from rabbit muscle as described by Bücher & Pfleiderer (1955), was dialysed against twice-distilled water and diluted so that the protein concentration was 1 mg./ml.

Radioactive amino acids. Uniformly labelled [^{14}C]glycine, L-[^{14}C]alanine, L-[^{14}C]leucine, L-[^{14}C]lysine and L-[^{14}C]-glutamic acid were obtained from The Radiochemical Centre, Amersham, Bucks.

Tissues. Spleen, liver and bone marrow were obtained from Wistar albino rats fed on stock diet. For spleen and liver, rats of 150 g. were used; for bone marrow rats of 250–350 g. were used. The bones of the limbs of the rats

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were prepared as free from muscle as possible, washed in cold saline and split with a forceps. The bone marrow was suspended with a glass rod in saline and washed twice. Rats bearing transplantable Murphy lymphosarcoma were obtained from the Imperial Cancer Research Fund Laboratories at Mill Hill, London.

Tissue preparations. (1) Cell-free suspensions. Spleen, liver and lymphosarcoma were homogenized with 2·5 vol. of a medium containing 0·01 M-$MgCl_2$, 0·03 M-$KHCO_3$, 0·025M-KCl, 0·02M-potassium phosphate buffer, pH 7·8, and 0·35 M-sucrose, in an all-glass Potter homogenizer, and packed bone-marrow cells were disrupted by freezing and thawing and then suspended in 2·5 vol. of the medium with the aid of an homogenizer. The suspensions were centrifuged at 2° for 10 min. at either 5000 *g* or 12 000 *g*.

(2) Microsomes. (*a*) By ultracentrifuging. The supernatants of tissue suspensions centrifuged at 12 000 *g* for 10 min. were spun in a model L Spinco preparative centrifuge (no. 40 rotor) for 50 min. at 105 000 $g_{av.}$. The microsome pellets were washed with 1 ml. of medium and suspended in 0·4 ml. of medium/g. of original tissue for liver, and in 0·4 ml. of medium/2 g. of original tissue for spleen, bone marrow and lymphosarcoma, by gentle homogenization. (*b*) By aggregation with Mg^{2+} ions. The supernatants of tissue suspensions in 0·35M-sucrose centrifuged at 12 000 *g* for 10 min. were brought to a $MgCl_2$ concentration of 0·02M and the aggregated microsomes were centrifuged for 10 min. at 12 000 *g*. The pellet was washed with a solution containing 0·35M-sucrose and 0·02M-$MgCl_2$, and was suspended by gentle homogenization in the medium in which the $MgCl_2$ was replaced by water, 0·4 ml. of the medium being used for 1 g. of original tissue wt. of liver, or 2 g. of spleen and lymphosarcoma.

(3) Mitochondria + microsomes. The supernatants of tissue suspensions centrifuged at 5000 *g* for 10 min. were spun in a model L Spinco preparative centrifuge (no. 40 rotor) for 50 min. at 105 000 $g_{av.}$. The pellet containing mitochondria + microsomes was washed with 1 ml. of medium and suspended by gentle homogenization in 0·8 ml. of medium/g. of original tissue for liver, and in 0·8 ml. of medium/2 g. of original tissue for spleen.

(4) Cell sap. Liver and spleen were homogenized in 2·5 vol. of 0·25M-sucrose and centrifuged for 50 min. in a model L Spinco preparative centrifuge (no. 40 rotor) at 105 000 $g_{av.}$. The supernatant was carefully pipetted out and used without dilution.

(5) pH 5 Fraction. The cell sap freed of microsomes either by ultracentrifuging or by $MgCl_2$ was brought to pH 5·2 by the addition of M-acetic acid. The precipitate ('pH 5 fraction') was centrifuged and suspended in one-fifth of the original volume of medium.

Method of incubation. The cell-free suspensions of isolated fractions were incubated under a continuous stream of $O_2 + CO_2$ (95:5) or $N_2 + CO_2$ (95:5) at 37° for 30 min. in a total volume of 1 ml. When mitochondria were present the incubation mixture contained 1 μmole of ATP, 1 μmole of FDP, 1 μmole of nicotinamide, mitochondria and labelled amino acid. When mitochondria were absent the incubation mixture contained 1 μmole of ATP, 10 μmoles of PEP and labelled amino acid. Where the cell sap was replaced by the pH 5 fraction 0·25 μmole of GTP and 0·05 ml. of PEP kinase solution were also added.

Isolation of proteins. If, after incubation, it was necessary to separate the different fractions the mitochondria were separated by centrifuging for 10 min. at 12 000 *g*, microsomes for 50 min. at 105 000 *g* and other particles ('post-microsomes') 180 min. at 105 000 *g*. The proteins were precipitated with aqueous 5% (w/v) trichloroacetic acid. They were then treated with hot trichloroacetic acid and organic solvents as described by Zamecnik, Loftfield, Stephenson & Steele (1951). In some cases, the normal washing procedure was followed by treatment of the protein with thioglycollic acid as reported by Siekevitz (1952) under the experimental conditions described by Campbell & Greengard (1959).

Measurement of radioactivity. The proteins were counted at infinite thickness on 0·28 cm.^{2} stainless-steel disks in a Tracerlab SC-16 windowless flow counter. The protein was compressed on the disk with a stainless-steel pestle as described by Busch, Simbonis, Anderson & Greene (1956). The counting procedure was as previously described (Campbell & Greengard, 1959).

RESULTS

Incorporation of different amino acids into nuclei-free suspensions

In metabolic studies on subcellular particles it is important to ensure that the preparations used do not contain whole cells. This is of particular importance with lymphatic tissues, in which the cells are difficult to disrupt. For this reason whole-tissue homogenates have not been employed in the present experiments. In every case the tissue suspension was first centrifuged at 5000 *g* for 10 min. and the sediment, which will also contain the nuclei, was rejected. This procedure also has the advantage of eliminating red blood cells present in the suspensions. Suspensions treated in this way are described as nuclei-free. The energy required for the incorporation of amino acids in this kind of tissue suspension containing mitochondria may be provided either by the intermediates of the Krebs cycle (see Siekevitz, 1952) or by intermediates in the glycolytic pathway (see Zamecnik & Keller, 1954). Since Wagle *et al.* (1958) used FDP, with spleen preparations, the same compound has been used in the present experiments. In preliminary experiments it was found that some of the conditions reported by Wagle *et al.* were not optimum for the incorporation of radioactivity. The incorporation was higher when the concentration of FDP was increased from 0·1 to 1·0 mM in the suspension and by the addition of mM-nicotinamide (see Zamecnik & Keller, 1954). The addition of coenzyme I, used by Emmelot (1957), did not enhance the incorporation. It can be seen from Table 1 that the suspensions prepared from the different lymphatic tissues, and from liver, incorporate labelled amino acids into their proteins by a mechanism which is stimulated by the addition of an energy source. With lymphosarcoma the effect of FDP was very small; the reason for this

small effect of FDP is not known. When nuclei-free suspensions were prepared from lung there was no significant incorporation of amino acids.

It will be seen that the activity of the spleen preparation for the incorporation of leucine was less than that of the liver preparation. In contrast Wagle *et al.* (1958) found that the incorporation of alanine, methionine and phenylalanine respectively was similar in preparations of spleen and liver. This suggested that a comparison of the incorporation of a range of different amino acids in preparations from the two types of tissue might be of interest. The results of such a comparison are shown in Table 2. It will be seen that the amino acids studied can be divided into two groups: in the first, containing glycine, alanine and glutamic acid, the incorporation in the presence of FDP into spleen is at least as great as into liver, whereas in the second group, containing lysine and leucine, the incorporation in the presence of FDP is much greater into liver than into spleen preparations.

Since glycine was used as one of the radioactive amino acids it was thought necessary to exclude the possibility that the results were affected by the synthesis of glutathione or similar peptides which could be bound to the proteins through –S–S– linkages. For this reason, the radioactive proteins were extracted with thioglycollic acid. As was found previously (Campbell & Greengard, 1959), this had no effect on the radioactivity of the proteins when leucine and lysine were used but considerably reduced the count for glycine. Wagle *et al.* (1958) used GTP in their experiments on the incorporation of amino acids into spleen suspensions. Keller & Zamecnik (1956) reported that in liver GTP is present in the cell sap and is needed only when purified preparations of the cell sap are used. It is still possible that spleen suspensions do not contain optimum amounts of GTP, but in our experiments reported in Table 2 the presence of GTP inhibited the incorporation of each of the different amino acids tested.

It can be seen from Table 2 that the incorporation of lysine into spleen suspensions is inhibited by the presence of FDP. The high control value in this case suggested that lysine was being incorporated by a non-energy-requiring mechanism, as has previously been observed (Borsook, Deasy, Haagen-Smit, Keighley & Lowy, 1950; Sarkar, Clarke & Waelsch, 1957), and it seemed possible that spleen contained a very active system for this type of incorporation. These authors have shown that the microsomes are not necessary for the incorporation of lysine into the proteins of the cell sap. Two separate enzyme systems appear to be involved in the incorporation, one which is enhanced by the presence of Ca^{2+} ions and inhibited by the presence of PO_4^{3-} ions and the other which is unaffected by the presence of these ions. It may be seen from Table 3 that when the cell sap is incubated with [^{14}C]lysine there is some incorporation of radioactivity into both tissue preparations, but that spleen is very much more active in this respect than liver. Whereas the incorporation into liver-cell sap was enhanced by the presence of Ca^{2+} ions, that into spleen-cell sap was unaffected. The presence of phosphate in the incubation mixture did not affect the incorporation into spleen-cell sap. The results of the experiments with [^{14}C]glutamic acid

Table 1. *Incorporation of labelled amino acids into the proteins of nuclei-free suspensions of liver, spleen, bone marrow and lymphosarcoma*

A sample (0·7 ml.) of the 5000 *g* supernatant (see Materials and Methods section) was incubated aerobically for 30 min. with 1 μmole of ATP, 1 μmole of nicotinamide and 1 μc of labelled amino acid, with and without 1 μmole of FDP. An equal volume of 10% trichloroacetic acid was then added. The results are expressed as radioactivity of the protein in counts/min. at infinite thickness/0·28 cm.^{2}.

Tissue	Labelled amino acid	Control	Plus FDP
Liver	Leucine	35	444
Spleen	Leucine	14	60
Bone marrow	Alanine	7	47
Lymphosarcoma	Leucine	37	51

Table 2. *Incorporation of various labelled amino acids into the proteins of nuclei-free suspensions of spleen and liver*

For incubation conditions and expression of the results see Table 1. Where GTP was added to the incubation mixture it was used as described in the Materials and Methods section. The proteins were treated with thioglycollic acid as described in the Materials and Methods section before their radioactivity was determined.

	Liver		Spleen		
Amino acid	Control	Plus FDP	Control	Plus FDP	Plus FDP plus GTP
Glycine	2	41	6	46	8
Alanine	7	71	48	68	28
Glutamic acid	14	27	19	67	25
Lysine	52	540	160	92	—
Leucine	91	381	32	82	73

and [^{14}C]leucine are included to show that no incorporation took place when these amino acids replaced lysine.

Effect of fructose diphosphate on the extent of incorporation

In the experiments reported by Zamecnik & Keller (1954) with liver suspensions the concentration of FDP used was 10 mM, whereas in the experiments discussed so far mM-FDP was used. An increase in the concentration of FDP from 1 to 10 mM doubled the incorporation of [^{14}C]alanine into liver preparations, but did not affect the incorporation of [^{14}C]leucine into spleen preparations (see Table 4). Table 4 also shows that, when such tissue preparations were incubated anaerobically with PEP as energy source, the incorporation of radioactivity was considerably less than with FDP under aerobic conditions.

Table 3. *Incorporation of radioactivity into proteins during the incubation of cell sap with* [^{14}C]*amino acids*

A sample (0·7 ml.) of the cell sap prepared as described in the Materials and Methods section was incubated anaerobically for 30 min. at 37°, with 1 μc of the labelled amino acid, and the final volume brought to 1 ml. with water. Ca^{2+} ions were added as $CaCl_2$ and phosphate as phosphate buffer, pH 7·6. In control experiments the reaction was stopped before incubation and the radioactivity of the proteins determined. Values obtained were subtracted from the experimental results to give the results in the table. Results are expressed as in Table 1.

Amino acid	Additions (20 mM)	Liver	Spleen
Lysine	—	8	42
Lysine	Ca^{2+} ions	32	47
Lysine	Phosphate	—	36
Leucine	Ca^{2+} ions	3	0
Glutamic acid	Ca^{2+} ions	0	1

Table 4. *Energy requirements for the incorporation of labelled amino acids into nuclei-free suspensions of liver and spleen*

A sample (0·7 ml.) of the 5000 *g* supernatants (prepared as described in the Materials and Methods section) was incubated with 1 μmole of ATP, 1 μc of labelled amino acid, [^{14}C]alanine for liver or [^{14}C]leucine for spleen and the additions indicated in the Table. In the presence of nicotinamide the incubation was carried out in an atmosphere of $O_2 + CO_2$ (95:5) and in the presence of PEP the atmosphere was $N_2 + CO_2$ (95:5). For expression of the results see Table 1.

Additions	Liver alanine	Spleen leucine
mM-Nicotinamide	6	10
mM-Nicotinamide plus mM-FDP	20	33
mM-Nicotinamide plus 10 mM-FDP	43	37
10 mM-PEP	17	17

Distribution of radioactivity in the subcellular fractions

Siekevitz (1952) has shown that when suspensions of disrupted liver cells are incubated with [^{14}C]-alanine and the various subcellular fractions are isolated by centrifuging the most active fraction for the incorporation of radioactivity is the microsomes. Siekevitz also found evidence for the aerobic formation of an 'activated alanine' by the mitochondria, which was transferred anaerobically to the microsomes. It was necessary therefore to determine whether the differences in the relative incorporation of the various amino acids into the protein of the tissue suspensions of the spleen and

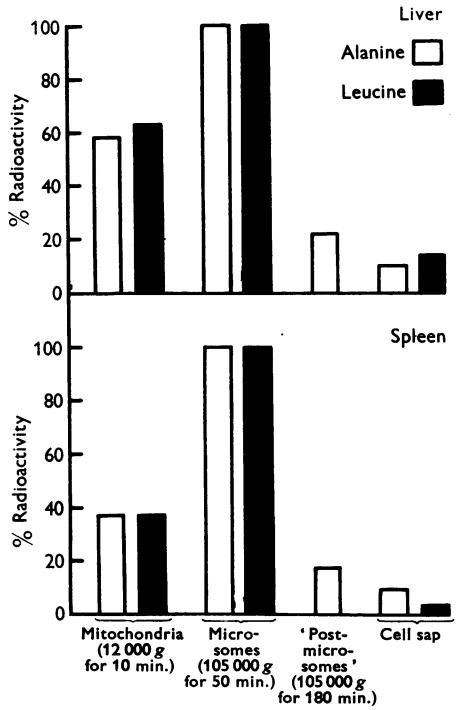

Fig. 1. Distribution of radioactivity in the different subcellular fractions of liver and spleen after incubation of suspensions in the presence of [^{14}C]alanine and [^{14}C]-leucine. The supernatant (2·1 ml.) obtained by centrifuging the liver suspension at 5000 *g* was incubated with 3 μc of labelled amino acid, 3 μmoles of FDP and 3 μmoles of nicotinamide. A similar supernatant (3·5 ml.) from spleen was incubated with 5 μc of labelled amino acid, 5 μmoles of FDP and 5 μmoles of nicotinamide. After incubation for 30 min. under $O_2 + CO_2$ (95:5) the various subcellular fractions were isolated as described in the Materials and Methods section. The proteins in the fractions were precipitated with 5% trichloroacetic acid and their radioactivity was determined. Results have been expressed as a percentage of the radioactivity of the microsomal fraction for each amino acid.

liver shown in Table 2 were due to differences in the intracellular distribution of the amino acid incorporation. In this and subsequent experiments alanine has been used as an example of the first group of amino acids and leucine of the second group. The results of the experiment shown in Fig. 1 indicate that in both tissues the incorporation of each amino acid occurs to the same extent in the microsomal and mitochondrial fractions. The incorporation of the amino acids into mitochondria compared with that of the microsomes is relatively less in spleen than in liver. The small particles that are sedimented on prolonged centrifuging, i.e. 'post-microsomes', are relatively inert compared with the microsomes. The specific activities of the microsomal proteins (respectively for liver 896 counts/min. with leucine, 75 with alanine; for spleen 366 with leucine, 369 with alanine) show again that whereas in spleen no great differences could be found for the extent of incorporation of the two labelled amino acids, a tenfold incorporation was found in liver with leucine instead of alanine.

Effect of cell sap on the incorporation of labelled alanine

As the mitochondrial fraction did not seem to be responsible for the differences of incorporation of alanine and leucine into liver preparations, the possibility that the cell sap could be responsible was considered. The incorporation of alanine has been followed with particulate fractions (mitochondria plus microsomes) of liver and spleen in the presence of cell sap from the two tissues. It has been found that the incorporation into liver proteins is enhanced when liver-cell sap is replaced by that of spleen (see Table 5), suggesting a greater incorporating ability of the spleen soluble cytoplasm for this amino acid. On the other hand, whereas spleen particles in the presence of spleen-cell sap fail to show an energy-dependent incorporation, these particles in the presence of liver-cell sap actively incorporate alanine.

Incorporation into nuclei- and mitochondria-free suspensions

The use of tissue suspensions freed of mitochondria by centrifuging of the tissue homogenate at 12 000 *g* for 10 min. was introduced by Zamecnik & Keller (1954), using liver suspensions. In the original experiments creatine phosphate was used as the energy donor, but in later experiments other energy-donor compounds such as PEP, 3-phosphoglyceric acid or carbamyl phosphate were used. Although spleen preparations were active also with 3-phosphoglyceric acid the experiments have been carried out with PEP as an energy donor. All the tissues used were found to incorporate labelled amino acids depending on the presence of an energy-donor compound (see Table 6). Again a similar preparation of lung failed to incorporate actively labelled alanine. In such systems it may be observed that whereas liver suspensions incorporate leucine more actively than a similar spleen suspension much smaller differences in the extent of incorporation are found with alanine as the labelled amino acid.

Incorporation into microsomal preparations

It seemed possible that the differences in the incorporation of alanine and leucine in the subcellular suspensions of spleen and liver could have been due to the presence of different amounts of the free amino acids in the preparations of the two tissues. The radioactive amino acids added to the incubation mixtures would then have been diluted to a different extent in the two cases, although the results reported in Table 5 made this explanation unlikely. In order to clarify the position it was desirable to use isolated microsome preparations with purified cell sap.

Table 5. *Incorporation of* [^{14}C]*alanine in liver and spleen subcellular particles (microsomes and mitochondria) in the presence of spleen- and liver-cell sap*

A suspension of the particles (0·4 ml.; prepared as described in the Materials and Methods section) was incubated for 30 min. at 37° with 0·3 ml. of the cell sap, 1 μmole of ATP, 1 μmole of nicotinamide and 1 μc of [^{14}C]alanine in the presence and absence of 1 μmole of FDP. For the expression of results see Table 1.

	Liver-cell sap		Spleen-cell sap	
Particles	Control	Plus FDP	Control	Plus FDP
Liver	19	41	44	103
Spleen	15	42	20	22

Table 6. *Incorporation of labelled amino acid into proteins of various tissue suspensions freed of nuclei and mitochondria*

A sample (0·7 ml.) of a 12 000 *g* supernatant (prepared as described in the Materials and Methods section) was incubated in an atmosphere of $N_2 + CO_2$ (95:5) for 30 min. at 37° with 1 μmole of ATP and 1 μc of labelled amino acid, with and without 10 μmoles of PEP. For the expression of the results see Table 1.

	Leucine		Alanine	
Tissue	Control	Plus PEP	Control	Plus PEP
Liver	3	331	6	31
Spleen	7	99	0	35
Bone marrow	—	—	14	57
Lymphosarcoma	17	115	—	—

Table 7. *Incorporation of labelled amino acids into microsomes plus cell sap or microsomes aggregated by Mg^{2+} ions plus pH 5 fraction, from different tissues*

In the experiments with the microsomes plus cell sap, the incubation mixture contained 0·4 ml. of microsome suspension and 0·3 ml. of cell sap, 1 μmole of ATP and 1 μc of labelled amino acid with and without PEP. In the experiments with the microsomes aggregated by Mg^{2+} ions plus pH 5 fraction, the incubation mixture contained 0·4 ml. of suspension of microsomes aggregated by Mg^{2+} ions, 0·3 ml. of pH 5 fraction, 1 μmole of ATP, 0·25 μmole of GTP and 2·5 μmoles of reduced glutathione, with and without 10 μmoles of PEP and PEP-kinase. For the expression of the results see Table 1.

	Microsomes prepared by ultracentrifuging plus cell sap				Microsomes prepared by aggregation with Mg^{2+} ions plus pH 5 fraction			
	Alanine		Leucine		Alanine		Leucine	
	Control	Plus PEP	Control	Plus PEP	Control	Plus PEP	Control	Plus PEP
Liver	12	134	6	1940	7	56	49	346
Spleen	30	155	78	425	10	46	22	50
Lymphosarcoma	16	48	12	109	15	32	17	42
Bone marrow	13	16	22	40	—	—	—	—

Table 8. *Incorporation of labelled alanine and leucine into microsome plus cell sap from liver and spleen*

The incubation mixture contained 0·4 ml. of microsome suspension, 0·3 ml. of cell sap, 1 μmole of ATP and 1 μc of labelled amino acid with and without 15 μmoles of PEP in the final volume of 1 ml. The incubation was carried out for 30 min. at 37° under an atmosphere of $N_2 + CO_2$ (95:5). For the expression of the results see Table 1.

	Liver-cell sap		Spleen-cell sap	
Microsomes	Control	Plus PEP	Control	Plus PEP
	Alanine			
Liver	12	134	21	214
Spleen	28	86	30	155
	Leucine			
Liver	6	1940	9	1734
Spleen	8	193	78	425

Microsomes were isolated from homogenates of both liver and various lymphatic tissues by ultracentrifuging. When such preparations were incubated with the cell sap derived from the tissue of origin an energy-dependent incorporation of radioactivity was obtained, as shown in Table 7. The preparations of bone marrow were less active than those of the other tissues and the incorporation of alanine in the extract from this tissue was not stimulated by the addition of PEP.

An alternative method for the preparation of active microsomes was provided by the work of Siekevitz (1952) and Gjessing, Floyd & Chanutin (1951). These authors showed that microsomes could be caused to aggregate by the addition to the tissue suspension of Mg^{2+} ions. Hoagland, Keller & Zamecnik (1956) and Keller & Zamecnik (1956) have shown that the fraction of the cell sap which is precipitated at pH 5·2 contains all the substances, with the exception of GTP, required for the incorporation of amino acids into microsomal proteins in the presence of an energy-generating system. Such a fraction has been incubated with the microsomes aggregated by Mg^{2+} ions and, as shown in Table 7, an energy-dependent incorporation of amino acid was obtained.

It may be observed that whereas the incorporation of [^{14}C]alanine is similar for both preparations from spleen and liver, the extent of incorporation of [^{14}C]leucine is very much greater in the preparations from liver than from spleen.

Effect of cell sap on the incorporation of amino acids into microsomes

As the incorporation of alanine into liver particles (mitochondria + microsomes) was greater in the presence of spleen-cell sap than liver-cell sap (Table 5), similar experiments have been carried out with microsomes obtained by ultracentrifuging from liver and spleen, alanine or leucine being used separately. In Table 8 it is seen that, if incorporation in the presence of PEP is considered, there is more incorporation with liver microsomes in the presence of spleen-cell sap than in the presence of liver-cell sap. However, with spleen microsomes, there is more incorporation in the presence of spleen-cell sap than in the presence of liver-cell sap. With labelled leucine and liver microsomes there is slightly less incorporation in the presence of spleen-cell sap than liver-cell sap, and with spleen microsomes there was more incorporation in the presence of spleen-cell sap than liver-cell sap.

The cell sap is a complex mixture of substances and it is possible that some compounds interfering with the stability of the microsomes could be present. It was therefore thought desirable to repeat these experiments with the partially purified cell sap (pH 5 fraction) instead of the whole-cell sap. The results are shown in Table 9. In this case, with either [^{14}C]leucine or [^{14}C]alanine the incorporation into both liver and spleen microsomes was greater in the presence of the pH 5

Table 9. *Incorporation of labelled alanine and leucine into microsomes plus* pH 5 *fraction from liver and spleen*

The incubation mixture contained 0·4 ml. of microsome suspension, 0·3 ml. of the pH 5 fraction, 1 μmole of ATP, 0·25 μmole of GTP and 1 μc of labelled amino acid, with and without 15 μmoles of PEP and PEP-kinase. Incubation was for 30 min. at 37° under an atmosphere of $N_2 + CO_2$ (95:5). For the expression of the results see Table 1.

	Liver pH 5 fraction		Spleen pH 5 fraction	
Microsomes	Control	Plus PEP	Control	Plus PEP
		Alanine		
Liver	8	312	5	87
Spleen	6	36	2	11
		Leucine		
Liver	15	1828	8	997
Spleen	10	181	10	125

fraction of liver than that of spleen. The spleen pH 5 fraction had a very low activity for the incorporation of [^{14}C]alanine into spleen microsomes.

DISCUSSION

In any comparison of the extent of incorporation of amino acids by subcellular fractions of different tissues the isolation of the components of the cell presents a major problem. Since ultrasonic disruption of liver microsomes substantially reduces the ability of the particles to incorporate amino acids (P. N. Campbell, unpublished observations), it is necessary to ensure than in the process of disruption of the cell the endoplasmic reticulum and its ribonucleoprotein particles are not damaged. This being so, it is not surprising that it is difficult to prepare satisfactory suspensions from tissues which contain large quantities of connective tissue, since homogenization must then be prolonged. Among the tissues studied in the present experiments lung and lymphosarcoma present particular difficulties in this respect. In some tissues such as bone marrow it is difficult to disrupt the cells at all.

As was explained above, practical considerations required the removal of the nuclei from the cell suspensions before incubation. A further advantage in this procedure arises from the fact that liver nuclei have been shown to inhibit the incorporation of amino acids into liver microsomes (Campbell & Greengard, 1959). Two types of subcellular preparations have been used therefore: one containing mitochondria plus microsomes plus cell sap incubated anaerobically with FDP and the other containing microsomes plus cell sap incubated anaerobically with PEP.

In the suspensions containing mitochondria the influence of the proportion of mitochondria to the total protein content must be considered. Thus the liver suspensions contained 4·5 mg. of mitochondrial protein/ml. of suspension, whereas spleen contained 1·7 mg./ml., the concentration of total protein in each suspension being similar. On this basis, the liver suspension would be expected to be able to utilize more FDP than that of the spleen. This was, in fact, found to be so.

The hope that the lymphosarcoma, being a rapidly growing tissue, might provide subcellular fractions with a higher activity than normal tissues was not fulfilled. In fact, the mechanism for the incorporation of amino acids by the tumour tissue did not appear to differ from that of the normal lymphatic tissues. Nevertheless the tumour tissue does have the advantage over spleen in that it contains fewer red blood cells, the protein of which would be relatively inert metabolically.

It remains to discuss the significance to be attached to the differences found in the extent of incorporation of leucine and alanine by subcellular preparations of liver and lymphatic tissue. For the purpose of the present discussion, it is assumed that the incorporation of amino acids in these preparations of subcellular particles represents the synthesis of complete protein from amino acids without the mediation of peptides. Provided that the amino acid composition of the synthesized protein in the two different tissues is similar, then the ratio of the specific radioactivity of the free amino acids and the protein-bound amino acids should be independent of the nature of the amino acid being studied (Campbell & Halliday, 1957). The amino acid composition of the protein being synthesized in spleen- and liver-tissue fractions is, of course, not known, but that of the whole-tissue proteins is similar (Müting & Wortmann, 1954).

On the basis of the figures given for the concentration of free alanine and leucine of cat liver by Tallan, Moore & Stein (1954), the specific radioactivity of the free amino acids in the cell sap used in the incubation of the microsomes would be 3·8 c/mole for alanine and 7·1 for leucine. Since the leucine and alanine content of liver protein is similar (Müting & Wortmann, 1954) it seems unlikely that these differences in the specific activity of the precursor amino acids could account for the tenfold difference in the extent of incorporation of the two amino acids by liver preparations.

Estimations of the concentration of free alanine in spleen and liver by Campbell & Stone (1957) can be used to show that the specific radioactivity of the free alanine in the cell sap used for the incubation of the microsomes was 3·3 c/mole for spleen and 3·8 c/mole for liver (a figure which agrees well with that based on the results of Tallan *et al.* 1954). In the experiment in which spleen-cell sap replaced liver-cell sap the incorporation of alanine by liver

microsomes was increased. In view of the similarity in the specific radioactivity of the free alanine in the two cell saps this result cannot be attributed to such a factor. Furthermore, when the whole-cell sap was replaced by the pH 5 fraction, which presumably contains only a small proportion of the free amino acids of the whole-cell sap, the extent of the incorporation of leucine into the spleen and liver microsomes was unaffected.

It may be observed that with spleen microsomes incubated with alanine, replacement of the whole-cell sap by the pH 5 fraction reduced the extent of the incorporation of amino acid. This suggests that a factor required for the incorporation of alanine with spleen-cell sap is not precipitated at pH 5·2.

One further point emerges from the studies with cell sap and pH 5 fraction. When [^{14}C]leucine is incubated with spleen microsomes the incorporation is greater in the presence of spleen-cell sap than of liver-cell sap, whereas liver pH 5 fraction is more effective than that of spleen. It seems probable that in the process of precipitation at pH 5·2 the activity of an inhibitor in the liver-cell sap for the incorporation of leucine is lost.

Although it is not possible at present to understand fully the interplay of the various factors involved in the incorporation of amino acids into subcellular fractions, two conclusions appear to be warranted. First, the ability of the microsome preparations from different tissues to synthesize protein *in vitro* is limited. The extent of this limitation is not necessarily correlated with activity *in vivo*. For example, under optimum experimental conditions, liver microsomes incorporate a given amino acid more actively than spleen microsomes. Secondly, the extent of incorporation of a given amino acid into the microsomes is regulated by factors present in the cell sap. The nature of these regulating factors is the subject of a later paper (Rendi & Campbell, 1959).

SUMMARY

1. When tissue suspensions containing mitochondria and microsomes are prepared from rat spleen, bone marrow and transplanted Murphy lymphosarcoma and are incubated with fructose diphosphate under aerobic conditions, an energy-dependent incorporation of amino acids takes place.

2. Whereas the extent of incorporation of ^{14}C-labelled glycine, alanine and glutamic acid was of the same order in such preparations of liver and spleen, ^{14}C-labelled leucine and lysine were more actively incorporated in the preparations from liver.

3. When [^{14}C]lysine, but not the other amino acids tested, was incubated with spleen-cell sap, the isolated protein was radioactive. The labelling of this protein was only slightly affected by the presence of Ca^{2+} or PO_4^{3-} ions in the medium.

4. When suspensions of lymphatic tissues were freed of mitochondria and incubated under anaerobic conditions with phosphoenolpyruvate, [^{14}C]alanine and [^{14}C]leucine were incorporated.

5. Active microsomes could be obtained from these tissues by either ultracentrifuging or aggregation with Mg^{2+} ions. When the microsomes prepared from spleen or liver were incubated with cell sap and phosphoenolpyruvate, alanine was incorporated to about the same extent in the two preparations whereas leucine was more actively incorporated in the preparation from liver.

6. When liver-cell sap was replaced by spleen-cell sap the incorporation of [^{14}C]alanine into liver microsomes was enhanced whereas the incorporation of [^{14}C]leucine was only slightly affected. On the other hand, the replacement of spleen-cell sap by liver-cell sap reduced the incorporation of both amino acids into spleen microsomes.

7. When the fraction of the spleen-cell sap precipitated at pH 5·2 replaced a similar fraction from liver, the incorporation of both amino acids into spleen and liver microsomes was reduced.

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Transglucosidase Activity of Rumen Strains of *Streptococcus bovis*

2. ISOLATION AND PROPERTIES OF DEXTRANSUCRASE*

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Dextransucrase, the enzyme which converts sucrose into dextran and fructose, has been found in the cell-free culture fluids of various strains of *Leuconostoc mesenteroides* (Hehre, 1951; Tsuchiya *et al.* 1952). The properties of the isolated enzymes have been described in detail (Koepsell *et al.* 1953; Bailey, Barker, Bourne & Stacey, 1957*a*). A number of species of streptococci have also been shown to produce dextran from sucrose (Hehre, 1951). In these organisms, however, the enzyme responsible for dextran synthesis does not appear to have been isolated or examined. Recently it was shown that various strains of *Streptococcus bovis* produce dextran in sucrose cultures only in the presence of added carbon dioxide (Dain, Neal & Seely, 1956; Bailey & Oxford, 1958*a*, *b*). In *Leuconostoc* species, however, dextran production appears to be independent of added carbon dioxide (Bailey & Oxford, 1958*a*). In view of the lack of information on streptococcal dextransucrase and because of the interesting effect of carbon dioxide it was decided to isolate and examine *S. bovis* dextransucrase.

In isolating *Leuconostoc* dextransucrase the main problem has been to obtain a preparation of the enzyme entirely free from preformed dextran. Various authors (e.g. Tsuchiya *et al.* 1952; Neely, 1958) have used the cell-free culture fluid from a *L. mesenteroides* strain grown in 2% sucrose medium as a dextransucrase preparation. Bailey *et al.* (1957*a*) found that dextransucrase isolated from this type of culture contained much dextran and could not be freed from this dextran. A modified method (Bailey, Barker, Bourne & Stacey, 1957*b*) gave a dextransucrase containing much less dextran. In this case, however, the enzyme had only weak dextransucrase activity except in the presence of added acceptor sugar, when it produced oligosaccharides and very little dextran.

This paper describes the isolation and properties of *S. bovis* dextransucrase, including a comparatively dextran-free preparation which gave a good conversion of sucrose into dextran and fructose.

* Part 1: Bailey (1959).

EXPERIMENTAL

Organism. *S. bovis* (strain I; Bailey & Oxford, 1958*a*, *b*), isolated from the rumen of a cow feeding on red clover, was used.

Culture media. (1) Basal medium. The organism was normally grown in a Bacto Casitone–Bacto Yeast Extract medium (Bailey & Oxford, 1958*b*) containing either sucrose (A.R.; 4%, w/v) or glucose (A.R.; 2%, w/v). Cultures were maintained in both the sucrose and glucose media.

(2) Synthetic medium. Oxford (1958) developed a synthetic medium suitable for growth and dextran production with *S. bovis*. This medium contained the same concentration of sucrose or glucose as the basal medium. Both basal and synthetic media were adjusted to pH 7·0. All cultures were incubated at 37° in CO_2 unless stated otherwise.

Carbohydrates. Sucrose and glucose were A.R. grade. All sugars used were shown, by paper-chromatographic analysis, to be free from other sugars (less than 0·1% of impurity). Dextran was isolated by precipitation with ethanol (2 vol.) from the cell-free culture fluid of *S. bovis* (strain I) which had been grown on sucrose for 8 hr. (to pH 5·1). The dextran was purified by the usual methods, e.g. Bailey & Oxford (1958*b*). Isomaltose and isomaltotriose were isolated from a partial acid hydrolysate of the dextran by fractionation with aqueous ethanol after absorption on a charcoal–Celite column (Turvey & Whelan, 1957).