the toxic action of *N. flava* venom. The correlation found between lecithinase A activity and the inhibitory action against succinic dehydrogenase is consistent with the view of Braganca & Quastel (1953) that the lecithinase A action of cobra venom is responsible for the inhibition of succinic dehydrogenase, probably by disruption of the mitochondria. However, our results indicate that both these activities are incidental to the primary toxic action.

The method of preparation by non-denaturing techniques of a fraction (fraction 1) from *N. flava* venom which has most of the toxicity of the venom but little of the enzymic activities is felt to represent an improvement over the method of heating the venom at 100° for 15 min. (Braganca & Quastel, 1953). The fraction obtained is more active, and it seems that the use of a denaturing technique such as heating to simplify a mixture of proteins is biochemically less satisfactory. It is frequently assumed that if a mixture of toxic proteins such as a snake venom is heated and no loss in toxicity is detected, the proteins primarily responsible for the toxicity of the mixture have not been affected by the heat treatment. There is no proof of this assumption. There is no reason to assume that the product obtained by heating cobra venom at 100° for 15 min., whether more, less or equally toxic, is the same biochemically as the original venom. Work is being continued in this Laboratory with this fraction in an attempt to explain its toxicity.

SUMMARY

1. *Naja flava* (cobra) venom has been separated into two electrophoretically distinct fractions, one (fraction 1) four times as toxic as the other (fraction 2).

2. Fraction 1 is composed of two highly mobile, strongly basic proteins comprising 80% by weight of the venom; fraction 2, which makes up the remaining 20% of the venom, consists of three proteins of low mobility. This fraction gives a positive Molisch test for carbohydrate.

3. Fraction 1, which is four times as toxic as fraction 2, is much less potent than fraction 2 with respect to lecithinase A, adenosine triphosphatase, diphosphopyridine nucleotidase and ribonuclease activities, indicating that none of these enzyme activities is of prime importance toxicilogically.

4. Fraction 2 is also a much more potent inhibitor of succinic dehydrogenase than fraction 1. Thus although the lecithinase A activity may well be responsible for the inhibition of succinic dehydrogenase, neither action is of importance as a toxic mechanism.

5. Comparison of the adenosine triphosphatase, diphosphopyridine nucleotidase and ribonuclease activities of *Naja flava* venom with those of two *Crotalus* venoms (*C. t. terrificus* and *C. adamanteus*) revealed that *Naja flava* venom was less active in each case.

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The Effect of Various Metabolites on Incorporation *in vitro* of Labelled Amino Acids into Protein of Normal Rat Diaphragm

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Incorporation of ^{14}C from DL-[1-^{14}C]alanine or from uniformly ^{14}C-labelled L-alanine into protein of the normal rat diaphragm is depressed by addition to the medium of sodium pyruvate and, to a less extent, of glucose (Sinex, MacMullen & Hastings, 1952; Manchester & Young, 1958). Manchester & Young (1958) studied the incorporation of radioactive material from another ten labelled amino acids, but found no diminution of incorporation in the presence of glucose. In the presence of pyruvate, incorporation from several amino acids was slightly smaller, but with only alanine and aspartic acid was the diminution substantial. Sinex *et al.* (1952) considered the possibility that in the presence of pyruvate, either added to the medium or formed from glucose, a

transaminase system (Braunstein & Kritzmann, 1937) could produce unlabelled alanine which might dilute labelled alanine entering the tissue from the medium, and so give rise to a diminution of incorporation into protein of ^{14}C from the added [^{14}C]alanine. If this explanation is correct a smaller incorporation of ^{14}C from glutamic and aspartic acid might occur when glucose or pyruvate is added to the medium, since α-oxoglutarate and oxaloacetate can be formed in the tricarboxylic acid cycle. But a diminution of incorporation might not occur if the keto acids formed are confined to the mitochondrion or are otherwise not accessible to transaminase-enzyme systems.

We have investigated these questions and have sought to elucidate why incorporation of ^{14}C from alanine is much more reduced by addition of glucose or pyruvate than is that of any other amino acid.

MATERIALS AND METHODS

Radioactive materials. Radioactive materials were obtained from the Radiochemical Centre, Amersham, Bucks. In the first experiment, where DL-[1-^{14}C]alanine was incubated in buffer in which diaphragm tissue had previously been shaken, the alanine had a specific activity of about 4·4 mc/m-mole and a concentration of 0·3 μmole/ml. In the experiments with uniformly ^{14}C-labelled glucose (designated [U-^{14}C]glucose) and sodium pyruvate, and with *N*-butyl-*N'*-sulphanilylurea (carbutamide) and *N*-butyl-*N'*-toluene-*p*-sulphonylurea (tolbutamide), the DL-[1-^{14}C]-alanine and the [^{14}C]glucose and pyruvate were all diluted to a specific activity of about 65 μc/m-mole. L-[U-^{14}C]-Alanine, [1-^{14}C]glycine and L-[U-^{14}C]aspartic acid had a specific activity of about 800 μc/m-mole, and L-[U-^{14}C]-leucine of about 400 μc/m-mole. In the experiments with sodium [5-^{14}C]oxoglutarate both the [^{14}C]oxoglutarate and L-[U-^{14}C]glutamic acid had a specific activity of about 400 μc/m-mole, though in other experiments [^{14}C]glutamic acid was used at a specific activity of about 800 μc/m-mole. In experiments with $NaH^{14}CO_3$ about 8 μc was added to each flask. Since 2 ml. of medium contained 54 μmoles of $NaHCO_3$ and 20 ml. of gas phase contained 1 ml. of CO_2 (about 50 μmoles), and a complete distribution of ^{14}C between HCO_3^- ion and CO_2 may be assumed, the specific activity was about 80 μc/m-mole.

Medium. Bicarbonate buffer, pH 7·4 (Gey & Gey, 1936), gassed with $O_2 + CO_2$ (95:5, v/v), was used throughout. Except where otherwise stated, DL-alanine had a final concentration of 2·7 μmoles/ml., L-alanine, glycine, L-leucine, L-glutamic acid and L-aspartic acid 1 μmole/ml. Oxaloacetic acid and α-oxoglutaric acid were dissolved in N-NaOH (1/0·5 mol.prop.) and then diluted with buffer to a final concentration of 12·5 μmoles/ml. Glucose and sodium pyruvate were also used at this concentration. NaCl was added to all media not containing keto acids in such amount as to keep the [Na^+ ion] of the medium constant.

Insulin (crystalline insulin, Boots Pure Drug Co. Ltd., Nottingham) was dissolved in 1·7 mN-HCl to give a concentration of 10 units/ml. A volume (0·1 ml.) of insulin solution (or of 1·7 mN-HCl as control) was added to each flask to make a final concentration of 0·45 unit/ml.

Carbutamide and tolbutamide (Burroughs Wellcome and Co., London) were dissolved in the minimum volume of N-NaOH and diluted with Geys's buffer to a final concentration of 10 mM or mM. Similar quantities of NaOH were added to control solutions and all were gassed to bring the pH to 7·4.

Chromatography. In the experiment where DL-[^{14}C]-alanine was incubated with the fluid in which diaphragm tissue had previously been shaken, 5 μl. samples of the medium were withdrawn at 30 min. intervals and pipetted on to Whatman no. 1 paper. When the spot was dry 5 μl. of 0·4% solution of unlabelled DL-alanine was applied to the same place to act as carrier. Alanine was separated by chromtography with a phenol–ammonia–water system, and was eluted with water from the paper on to a 2 cm. diam. aluminium disk. The solution was dried as a thin film and the radioactivity assessed by the method of Boursnell, Coombs & Rizk (1953). Since the quantities of alanine were very small no correction for self-absorption was required.

Fixation of carbon dioxide. Warburg manometer flasks were used for experiments with $NaH^{14}CO_3$. Each flask contained 2 ml. of Geys's buffer in the centre portion and 8 μc of $NaH^{14}CO_3$ in 0·1 ml. of 0·01 N-NaOH in the side arm. The diaphragm was prepared as before and transferred to Geys's buffer. The side arm was then immersed in a freezing mixture to freeze the solution in the side arm whilst the flask was gassed with $O_2 + CO_2$ or $N_2 + CO_2$ and sealed. The contents of the side arm were melted and added to the diaphragm, and the flasks incubated.

Preparation of diaphragm. A female albino Wistar rat, which weighed 100–150 g. and had fasted 20–24 hr. before use, was decapitated and bled and the diaphragm removed. The two halves of the diaphragm were washed in freshly gassed buffer, and then gently blotted and transferred to small conical flasks containing 2 ml. of medium. As far as possible one half of a diaphragm was used as control for the other half. Two hemidiaphragms were added to each flask. The flasks were gassed, sealed and incubated at 37° in a Dubnoff shaker.

Preparation of protein from diaphragm. After incubation for 2 hr. the diaphragm was removed, and blotted and homogenized in a Potter–Elvehjem homogenizer in 10 ml. of 10% (w/v) trichloroacetic acid. The precipitate was spun down, resuspended in 10 ml. of 10% trichloroacetic acid and heated for 15 min. at 90°. The insoluble material was extracted twice with 1·5 ml. of 0·4 N-NaOH, and the insoluble residue rejected (protein content about 5%). The dissolved protein was reprecipitated by the addition of 5 ml. of 10% trichloroacetic acid. It was washed with 2 ml. of ethanol–ether (50:50, v/v), and then with 2 ml. of ether and was dried in a vacuum desiccator.

Assessment of radioactivity of diaphragm protein. Two hemidiaphragms, combined weight about 200 mg., yielded by the method described 25–30 mg. of protein which had a nitrogen content of about 12·5% (consistent with a protein content of about 80%), and about 0·05% of phosphorus. The samples of protein were ground in a mortar, collected on 1 $cm.^2$ plastic disks, and counted as infinitely thick samples (Popják, 1950), a thin end-window Geiger–Müller tube being used. Sufficient counts were recorded to give a counting error of less than 5%.

Statistical treatment of results. Experiments dealing with [^{14}C]-glucose and -pyruvate and with carbutamide and tol-

butamide were performed in groups of six, one each with and without addition of insulin or carbutamide or tolbutamide in the absence of added substrate, with and without addition of insulin or carbutamide or tolbutamide in the presence of glucose, and with and without addition of insulin or carbutamide or tolbutamide in the presence of pyruvate. Counts found were expressed as a percentage of the counts of the control. This procedure was repeated four or six times. The percentages so obtained were grouped, averaged and the standard errors determined. Significance was assessed on the basis of Student's *t* test.

In most other experiments incorporation in the presence of various additions to the medium was expressed as a percentage of the control value for incorporation in the absence of these additions. Percentages so obtained were grouped, averaged and the standard errors determined. Significance was assessed on the basis of Student's *t* test.

RESULTS

Alanine. That the peculiar behaviour of alanine in the presence of glucose or pyruvate (Sinex *et al.* 1952; Manchester & Young, 1958) is not due to destruction of alanine outside the tissue is indicated by the results in Table 1. When [^{14}C]alanine is shaken in a pyruvate-containing medium in which diaphragm tissue has been incubated for 1 hr. and then removed, the amount of alanine does not fall. Such an experiment does not preclude the possibility that the specific activity of the alanine falls because of the appearance of unlabelled alanine in the medium.

Table 1. *Effect on* DL-[1-^{14}C]*alanine of incubation in a medium containing pyruvate and in which diaphragm tissue has previously been shaken*

Time of incubation of [^{14}C]alanine (hr.)	Counts/min. from [^{14}C]alanine separated by paper chromatography from 5 μl. of incubating medium	
	Expt. 1	Expt. 2
0	965	986
0·5	931	963
1·0	884	991
1·5	911	913
2·0	—	935
2·5	979	990

Bornstein (1957) observed that carbutamide and tolbutamide *in vitro* inhibited a liver-transaminase system involving alanine and oxoglutarate. If the diminution of incorporation of ^{14}C from alanine into protein of normal diaphragm in the presence of glucose or pyruvate is the result of the dilution of the labelled alanine by unlabelled alanine formed from carbohydrate by transamination (Sinex *et al.* 1952; Manchester & Young, 1958), incorporation of ^{14}C might be stimulated in the presence of carbutamide or tolbutamide. In the presence of these substances formation of alanine from pyruvate would be less than in their absence, and the specific activity of alanine inside the tissue would be higher. However, we have observed that addition of carbutamide and tolbutamide to the medium results in incorporation of ^{14}C from DL-[^{14}C]alanine to a less extent than is observed in the absence of these substances. This was true when glucose or pyruvate was present in the medium, as well as in their absence (Table 2).

On the other hand, when [U-^{14}C]glucose and [1-^{14}C]pyruvate were present in the medium together with DL-[1-^{14}C]alanine, the glucose and pyruvate having the same specific activity as the added DL-alanine, incorporation of ^{14}C was substantially higher in the presence of pyruvate (Table 3). Addition of insulin under these conditions promoted still further the incorporation of ^{14}C into diaphragm protein (Tables 4, 5; Fig. 1).

Table 2. *Effect of the addition of carbutamide or tolbutamide on the incorporation* in vitro *of* ^{14}C *from* DL-[1-^{14}C]*alanine into protein of normal rat diaphragm*

Results are expressed as a percentage of the control value found in the absence of added carbutamide or tolbutamide. The value of *P* for a difference which is significant is given in brackets. Number of observations is given in parentheses.

Effect of the addition of	Medium including: No added substrate. Radioactivity in diaphragm protein		Glucose. Radioactivity in diaphragm protein		Pyruvate. Radioactivity in diaphragm protein	
	Control experiments (counts/min./disk)	Percentage of control value in the presence of added carbutamide or tolbutamide	Control experiments (counts/min./disk)	Percentage of control value in the presence of added carbutamide or tolbutamide	Control experiments (counts/min./disk)	Percentage of control value in the presence of added carbutamide or tolbutamide
Carbutamide (10 mM)	93	82·0±4·3 (4)	75	90·0±4·1 (4)	50	87·0±5·2 (4)
Tolbutamide (10 mM)	91	9·1±1·8 (4) [P<0·001]	70	8·2±0·8 (4) [P<0·001]	52	21·6±1·7 (4) [P<0·001]
Tolbutamide (mM)	105	94·0±2·6 (4)	88	99·2±2·2 (4)	59	93·2±1·0 (4)

Effect of oxoglutarate. In view of the results obtained with alanine and the related keto acid, pyruvate, it was of interest to study the effect of addition to the medium of α-oxoglutarate on the incorporation of ^{14}C from amino acids and, in particular, that from glutamic acid. Incorporation of ^{14}C from [^{14}C]glutamic acid was substantially depressed when oxoglutarate was added to the medium (Table 6). Addition of oxoglutarate also diminished, though to a much less extent, incorporation of ^{14}C from [^{14}C]-glycine and -leucine (Table 6). When [5-^{14}C]oxoglutarate was present in the medium together with L-[U-^{14}C]glutamic acid, the oxoglutarate having the same specific activity as the added L-glutamic acid, incorporation of ^{14}C was much higher in the presence of oxoglutarate (Table 7). However, addition of oxoglutarate did not depress significantly incorporation into protein of ^{14}C from either alanine or aspartic acid; on the contrary, under these conditions incorporation of ^{14}C from aspartic acid was somewhat enhanced (Table 6).

Effect of oxaloacetate. As with α-oxoglutarate, addition of oxaloacetate markedly depressed incorporation of ^{14}C into protein from glutamic acid and slightly diminished, though to a much less extent, incorporation of ^{14}C from glycine and leucine (Table 8). Also like oxoglutarate, addition

Table 3. *Effect of the addition of* [^{14}C]*glucose or* [^{14}C]*pyruvate to a medium containing* DL-[1-^{14}C]*alanine on the incorporation* in vitro *of* ^{14}C *into protein of normal rat diaphragm*

No insulin was added to the medium. Results are expressed as a percentage of the control value found in the absence of added glucose or pyruvate. The value of P for a difference which is significant is given in brackets. Number of observations is given in parentheses.

Radioactivity in diaphragm protein		
Control experiments (counts/min./disk)	Percentge of control value in the presence of DL-[1-^{14}C]alanine and of added	
	[^{14}C]Glucose	[^{14}C]Pyruvate
66	187·8±19·3 (6) [P<0·01]	211·3±14·3 (6) [P<0·001]

Table 4. *Effect of the addition of insulin* (0·5 *unit/ml.*) *to a medium containing* DL-[1-^{14}C]*alanine without added oxidizable substrate or with* [^{14}C]*glucose or* [^{14}C]*pyruvate, on the incorporation* in vitro *of* ^{14}C *into protein of normal rat diaphragm*

Results are expressed as a percentage of the control value found in the absence of added insulin. The value of P for a difference which is significant is given in brackets. Number of observations is given in parentheses.

Medium including					
No added substrate. Radioactivity in diaphragm protein		[^{14}C]Glucose. Radioactivity in diaphragm protein		[^{14}C]Pyruvate. Radioactivity in diaphragm protein	
Control experiments (counts/min./disk)	Percentage of control value in the presence of insulin	Control experiments (counts/min./disk)	Percentage of control value in the presence of insulin	Control experiments (counts/min./disk)	Percentage of control value in the presence of insulin
66	136·2±7·9 (6) [P<0·01]	117	158·3±6·7 (6) [P<0·001]	132	136·3±7·2 (6) [P<0·01]

Table 5. *Effect of the addition of* [^{14}C]*glucose or* [^{14}C]*pyruvate to a medium containing both insulin and* DL-[1-^{14}C]*alanine on the incorporation* in vitro *of* ^{14}C *into protein of normal rat diaphragm*

Insulin (0·5 unit/ml.) was present in each experiment. Results are expressed as a percentage of the control value found in the absence of added glucose or pyruvate. The value of P for a difference which is significant is given in brackets. Number of observations is given in parentheses.

Radioactivity in diaphragm protein		
Control experiments (counts/min./disk)	Percentage of control value in presence of added	
	[^{14}C]Glucose	[^{14}C]Pyruvate
91	218·7±19·1 (6) [P<0·01]	219·3±29·2 (6) [P=0·01]

of oxaloacetate did not significantly depress incorporation of ^{14}C from aspartic acid, but addition of oxaloacetate did diminish incorporation into protein of ^{14}C from alanine (Table 8; Fig. 2).

Fixation of carbon dioxide. We have found that when normal rat diaphragm is incubated in a $H^{14}CO_3^-$ buffer, ^{14}C is incorporated into protein (Table 9). Furthermore, this incorporation of ^{14}C into protein is stimulated by addition of pyruvate to the medium. When the gas phase is changed from $O_2 + CO_2$ to $N_2 + CO_2$ incorporation of ^{14}C from $NaH^{14}CO_3$ into protein is almost completely inhibited.

DISCUSSION

Effect of size of amino acid pool on rate of incorporation of ^{14}C from [^{14}C]amino acids

Incorporation of ^{14}C from both [^{14}C]-glutamic and -aspartic acid is much less than from other amino acids. Since free glutamic acid has been found in muscle in large quantities, a possible explanation of the poor incorporation of ^{14}C from glutamic acid is that the dilution of specific activity of [^{14}C]amino acids which takes place when they enter the amino acid pool in the muscle cell is greater for glutamic acid than for other amino acids. In addition, a portion of the [^{14}C]-glutamic and -aspartic acid may be converted into glutamine and asparagine before incorporation into protein. The specific activity of [^{14}C]-glutamine and -asparagine would therefore be less than that of added [^{14}C]-glutamic and -aspartic acid as a result of two dilutions. However, semi-quantitative experiments by us in which the free amino acids of fresh rat diaphragm have been separated by paper chromatography indicate that both glycine and alanine, as well as glutamic acid, are present in amounts considerably greater than those of other amino acids, and that

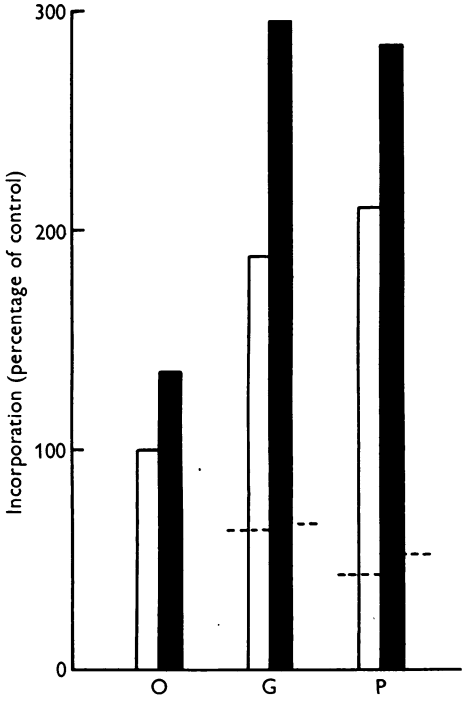

Fig. 1. Effect of addition of insulin, [^{14}C]glucose and [^{14}C]pyruvate to a medium containing [^{14}C]alanine on the incorporation *in vitro* of ^{14}C into protein of normal rat diaphragm. Figures on the ordinate express incorporation as a percentage of that found in the absence of insulin, glucose and pyruvate. Open blocks represent incorporation in the absence of insulin, solid blocks in the presence of insulin. O signifies that no oxidizable substrate had been added to the medium, G that [^{14}C]glucose was present and P that [^{14}C]pyruvate was present. Broken lines indicate the results obtained by Manchester & Young (1958) with unlabelled glucose and pyruvate.

Table 6. *Effect of the addition of α-oxoglutarate to the medium on the incorporation* in vitro *of ^{14}C from amino acids into protein of normal rat diaphragm*

Results are expressed as a percentage of the control value found in the absence of added oxoglutarate. The value of P for a difference which is significant is given in brackets. Number of observations is given in parentheses.

	Radioactivity in diaphragm protein	
[^{14}C]Amino acid added to medium	Control experiments (counts/min./disk)	Percentage of control value in the presence of added oxoglutarate
L-Alanine	691	90·7±4·2 (6)
Glycine	448	80·5±5·4 (6) [$P<0.02$]
L-Leucine	967	92·7±2·5 (6) [$P<0.05$]
L-Glutamic acid	105	50·3±3·3 (6) [$P<0.001$]
L-Aspartic acid	114	123·8±10·3 (6)

Table 7. *Effect of the addition of [^{14}C]α-oxoglutarate to a medium containing [^{14}C]glutamic acid on the incorporation* in vitro *of ^{14}C into protein of normal rat diaphragm*

Results are expressed as a percentage of the control value found in the absence of added [^{14}C]oxoglutarate. The value of P for a difference which is significant is given in brackets. Number of observations is given in parentheses.

Radioactivity in diaphragm protein	
Control experiments (counts/min./disk)	Percentage of control value in the presence of [^{14}C]α-oxoglutarate
28·5	403±16 (6) [$P<0.001$]

there appears to be little aspartic acid. Approximate figures (mg./100 g.) obtained by Tallan, Moore & Stein (1954) for the free amino acids of cat muscle are 30 for glutamic acid, 20 for alanine and for glycine, 5 for aspartic acid, 5 or below for other amino acids. Since incorporation of ^{14}C from both alanine and glycine is several times that from glutamic acid and aspartic acid, despite the relatively large pool of free alanine and glycine in the cell, reduction of specific activity is probably not alone responsible for the smallness of incorporation of ^{14}C from glutamic and aspartic acids when these substances enter the cell, although it almost certainly contributes to it. Probably a more important cause is that even in the absence of added substrate a portion of the [^{14}C]glutamic acid or [^{14}C]aspartic acid is transformed into a compound or compounds which cannot be incorporated into protein. It is perhaps not surprising that the two acids which give rise to least incorporation are the two most active in transamination (Braunstein, 1947).

Effect of transamination reactions on incorporation of ^{14}C from [^{14}C]amino acids

Braunstein & Kritzmann (1937) first showed that interconversion of amino and keto acids could take place by a process of transamination without the intermediate formation of ammonia. They studied three transaminase systems, namely glutamic acid–aspartic acid, glutamic acid–alanine and aspartic acid–alanine. Though subsequent work has shown that nearly all amino acids can take part in transamination reactions, the glutamic acid, aspartic acid and alanine systems are still considered as being the most active. For example, Awapara & Seale (1952) found that the formation of glutamic acid from oxoglutarate in a homogenate of rat-skeletal muscle, when aspartic acid or alanine was added, was over ten times that in the presence of leucine, isoleucine or valine. However, the glutamic, aspartic and alanine systems are believed not to be of similar activity. Braunstein (1947) states that in animal tissues the glutamic acid–aspartic acid transaminase enzyme is more active than the glutamic acid–alanine enzyme,

Table 8. *Effect of the addition of oxaloacetate to the medium on the incorporation* in vitro *of ^{14}C from amino acids into protein of normal rat diaphragm*

Results are expressed as a percentage of the control value found in the absence of added oxaloacetate. The value of P for a difference which is significant is given in brackets. Number of observations is given in parentheses.

[^{14}C]Amino acid added to medium	Radioactivity in diaphragm protein: Control experiments (counts/min./disk)	Radioactivity in diaphragm protein: Percentage of control value in the presence of added oxaloacetate
L-Alanine	593	59·2±2·8 (6) [$P<0·001$]
Glycine	638	89·5±4·1 (6) [$P=0·05$]
L-Leucine	1220	88·2±3·0 (6) [$P<0·02$]
L-Glutamic acid	157	40·7±1·9 (6) [$P<0·001$]
L-Aspartic acid	107	105·5±4·1 (12)

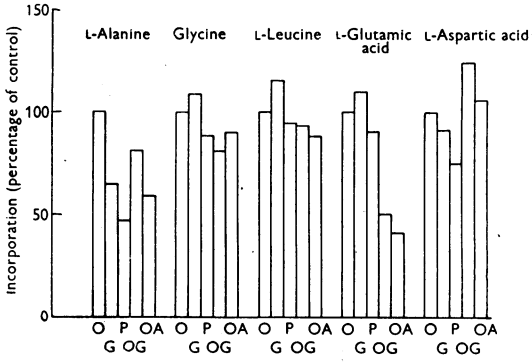

Fig. 2. Effect of addition to the medium of glucose, pyruvate, α-oxoglutarate and oxaloacetate on the incorporation *in vitro* of ^{14}C from [^{14}C]amino acids into protein of normal rat diaphragm. Figures on the ordinate express incorporation as a percentage of that found in the absence of added substrates. O signifies that no oxidizable substrate had been added to the medium, G, P, OG and OA that glucose, pyruvate, oxoglutarate or oxaloacetate respectively was present. Figures for the effect of glucose and pyruvate are taken from Manchester & Young (1958).

Table 9. *Effect of anaerobiosis and of addition of pyruvate on the incorporation* in vitro *of ^{14}C from $NaH^{14}CO_3$ into protein of normal rat diaphragm*

Number of observations is given in parentheses. The value of P is given where a difference between means is significant.

	Radioactivity in diaphragm protein (counts/min./disk)	
(1) No addition to medium		
(*a*) Gas phase O_2+CO_2 (95:5)	53·4±5·1 (3)	$P<0·001$
(*b*) Gas phase N_2+CO_2 (95:5)	4·0±1·1 (3)	
(2) Gas phase O_2+CO_2 (95:5)		
(*a*) No addition to medium	42·9±1·6 (6)	$P<0·02$
(*b*) Addition of pyruvate	49·5±1·7 (5)	

which in turn is more active than the aspartic acid–alanine system. This conclusion was confirmed by Awapara & Seale (1952), with a heart-muscle homogenate.

Transamination between [^{14}C]amino acids and unlabelled keto acids could lead to a diminution in incorporation of ^{14}C into protein by reducing the quantity of [^{14}C]amino acid available for protein synthesis. Moreover formation of unlabelled amino acid from added keto acid by transamination would depress incorporation of ^{14}C from [^{14}C]amino acid of the same kind. Other considerations apart, the more active the transaminating system and the more keto acid available, the smaller is the incorporation of ^{14}C from [^{14}C]amino acid to be expected.

Nisonoff, Barnes, Enns & Schuching (1954) showed that transamination could take place between an amino acid and the corresponding keto acid. The occurrence of such a process in diaphragm between [^{14}C]alanine and unlabelled pyruvate, as well as the formation of unlabelled alanine from pyruvate and other amino acids, would explain the diminution of incorporation of ^{14}C from alanine into protein which has been observed when pyruvate is added to the medium (Sinex *et al.* 1952; Manchester & Young, 1958). Addition of glucose instead of pyruvate also diminished incorporation from alanine, presumably because of the breakdown of glucose to pyruvate within the cell. Substances which inhibit transamination might therefore lessen the diminution of incorporation of ^{14}C from alanine which occurs when glucose or pyruvate is added to the medium.

The observation that addition of carbutamide or tolbutamide, which Bornstein (1957) found to inhibit a liver-transaminase system, fails to stimulate incorporation *in vitro* of ^{14}C from alanine in the presence of glucose or pyruvate might be taken as some evidence against the idea that the diminution of incorporation of ^{14}C in the presence of glucose and pyruvate is due to a dilution of [^{14}C]alanine by unlabelled alanine formed from pyruvate by transamination. On the other hand, it is possible that neither carbutamide nor tolbutamide inhibits muscle transaminase, as opposed to liver transaminase, or that these substances have other actions which result in an inhibition of incorporation. Incorporation of ^{14}C from [^{14}C]amino acid is greatly depressed by many well-known metabolic inhibitors (K. L. Manchester & F. G. Young, unpublished work), and it is possible that carbutamide and tolbutamide inhibit the process of incorporation at concentrations lower than those at which they can exert any putative effect on transaminase-enzyme systems.

Effect of [^{14}C]*-glucose and -pyruvate.* The observation that ^{14}C from [^{14}C]-glucose and -pyruvate is incorporated into protein under the conditions of our experiments (Fig. 1) agrees with the idea that transamination takes place in rat diaphragm. The only [^{14}C]amino acids likely to be formed from [1-^{14}C]pyruvate are alanine and, possibly, serine and glycine, since any amino acids formed by reactions involving preliminary decarboxylation of pyruvate would be unlabelled. Thus aspartic acid or glutamic acid formed from [1-^{14}C]pyruvate by operation of the tricarboxylic acid cycle would be unlabelled unless the $^{14}CO_2$ liberated in the cycle was subsequently fixed into oxaloacetate. No such limitations would apply, however, to [U-^{14}C]-glucose, and it is possible that [^{14}C]alanine, glutamic acid and aspartic acid are formed from [^{14}C]glucose, unless, as is argued below, the appropriate keto acids formed from glucose, that is oxoglutarate and oxaloacetate, are generated in a locus which renders them not readily available to transaminase enzymes.

Effect of oxoglutarate. Manchester & Young (1958) found that although addition of glucose and pyruvate depressed incorporation into protein of ^{14}C from alanine, these substrates did not depress incorporation of ^{14}C from glutamic acid, an occurrence which might have been expected if transamination was taking place. They considered the possibility that because the enzymes of the tricarboxylic acid cycle are situated within the mitochondrion, oxoglutarate formed by oxidation of glucose or pyruvate might not be available for transamination by enzymes outside the mitochondrion. Free keto acids are present in tissues in only small quantities, and it is perhaps significant that Cavallini & Frontali (1954) found in rat muscle three times as much pyruvate as oxoglutarate (0·3 and 0·1 mg./100 g. respectively).

Addition of unlabelled oxoglutarate depresses incorporation of ^{14}C from glutamic acid (Fig. 2). Clearly, under these conditions [^{14}C]glutamic acid is diluted in the cell either by transamination with unlabelled oxoglutarate to form unlabelled glutamic acid, or by formation of glutamic acid from added oxoglutarate and, say, alanine from the cell pool, or by a combination of both processes. That unlabelled glutamic acid is being formed from added oxoglutarate by transamination is suggested by the observation that incorporation of ^{14}C from glycine and leucine is slightly depressed when oxoglutarate is added.

Incorporation of ^{14}C from [5-^{14}C]oxoglutarate could result from two possible processes. One of these is the formation of [^{14}C]glutamic acid by transamination or by the glutamic dehydrogenase reaction (Dewan, 1938). The second possible process is the production of [^{14}C]aspartic acid from [^{14}C]oxaloacetate which had itself been formed by the oxidation of [^{14}C]oxoglutarate in the tricarboxylic acid cycle.

In view of these possibilities, it is surprising that addition of oxoglutarate does not depress incorporation of ^{14}C from either aspartic acid or alanine under the conditions of our experiments. For this fact we have at present no adequate explanation.

Effect of oxaloacetate. The addition of oxaloacetate depresses incorporation into protein of ^{14}C from glutamic acid and, to a much less extent, from glycine and leucine (Fig. 2), presumably by a process of transamination in which aspartic acid and [^{14}C]keto acids are formed. Since the [^{14}C]keto acids cannot be incorporated into protein as such, incorporation of ^{14}C is depressed.

Addition of oxaloacetate, unlike addition of oxoglutarate, depresses incorporation of ^{14}C from alanine (Fig. 2), though whether this is due to the addition of oxaloacetate itself or to the formation of pyruvate from oxaloacetate by decarboxylation inside the cell, is not certain. As with oxoglutarate, however, addition of oxaloacetate does not depress incorporation of ^{14}C from aspartic acid (Fig. 2), and again no satisfactory explanation can at present be offered.

The behaviour of aspartic acid is peculiar in several ways. Apart from alanine, it is the only amino acid from which incorporation of ^{14}C is depressed by addition of pyruvate, and yet incorporation of ^{14}C from aspartic acid is not diminished in the presence of oxoglutarate or oxaloacetate. Incorporation of ^{14}C from aspartic acid is extremely low despite the fact that the cell pool appears to be small, and the [^{14}C]acid may therefore suffer only a relatively small dilution on entering the cell.

Incorporation of $^{14}CO_2$

Fixation of carbon dioxide by pyruvate to form oxaloacetate in animal tissues was first demonstrated by Krebs & Eggleston (1940) in minced pigeon liver. Ochoa (1945), using an extract of washed acetone-dried pig heart, showed that the enzymic decarboxylation of oxalosuccinate to oxoglutarate and carbon dioxide was reversible. Stern (1948), using a wide variety of animal tissues, was able to demonstrate fixation of carbon dioxide into oxaloacetate in only pigeon liver and, to a smaller extent, sheep-kidney cortex, though many tissues formed citric acid from oxoglutarate. In our experiments the entry of ^{14}C from $NaH^{14}CO_3$ into diaphragm protein is strong evidence that fixation of carbon dioxide can take place in mammalian muscle. Moreover, incorporation of ^{14}C into protein indicates that fixation into oxaloacetate is almost certainly involved with the subsequent formation of [^{14}C]aspartic acid. Fixation of carbon dioxide by oxoglutarate or oxalosuccinate would not result in the formation of [^{14}C]glutamic acid or [^{14}C]aspartic acid, and would therefore not provide for the formation of [^{14}C]protein.

SUMMARY

1. Incorporation into rat-diaphragm protein *in vitro* of ^{14}C from a medium containing [^{14}C]alanine is greater when [^{14}C]-glucose or -pyruvate is present in the medium than in their absence. Addition of insulin further increases incorporation of ^{14}C in both the presence and the absence of [^{14}C]-glucose or -pyruvate.

2. Addition of oxoglutarate to the medium substantially depresses incorporation into protein of ^{14}C from [^{14}C]glutamic acid, and slightly depresses that from alanine, glycine and leucine. Incorporation of ^{14}C from aspartic acid is somewhat enhanced under these conditions.

3. Incorporation into diaphragm protein of ^{14}C from a medium containing [^{14}C]glutamic acid is vastly increased when [^{14}C]oxoglutarate is also added.

4. Addition of oxaloacetate depresses severely incorporation into protein of ^{14}C from [^{14}C]-alanine and -glutamic acid, and slightly from glycine and leucine. Incorporation of ^{14}C from aspartic acid is not affected.

5. In the presence of $NaH^{14}CO_3$, ^{14}C is incorporated into protein of normal rat diaphragm.

6. Incorporation into protein of normal rat diaphragm of ^{14}C from [^{14}C]alanine is depressed in the presence of carbutamide or tolbutamide whether or not glucose or pyruvate is present.

7. The results are largely, but not entirely, explicable on the assumption that transamination reactions, together with carboxylation, can proceed in isolated rat diaphragm under the conditions of our experiments.

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The Labelling by [^{14}C]Amino Acids of Cell-Sap Protein in a Cell-Free System from Guinea-pig Liver

THE SITE OF ORIGIN OF LABELLED PROTEIN

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It is now established that amino acids are incorporated into the protein of the microsome fraction of tissues such as liver at a more rapid rate than into the protein of other subcellular fractions both in the intact animal (e.g. Borsook, Deasy, Haagen-Smit, Keighley & Lowy, 1950; Hultin, 1950; Keller, 1951) and in cell-free systems (e.g. Siekevitz, 1952; Zamecnik & Keller, 1954). These findings suggest that the microsome fraction is of importance as a site of synthesis of other cytoplasmic proteins. While no evidence has been obtained showing that the microsome fraction is the site of origin of all cytoplasmic proteins, some evidence has been obtained which indicates that it is the site of synthesis of certain specific soluble proteins (e.g. Peters, 1957; Rabinovitz & Olson, 1956). It would be therefore of great value if the synthesis of specific soluble proteins could be studied in entirely cell-free systems. However, few instances have been reported of an unequivocal demonstration of either the labelling or net synthesis of a specific soluble protein in a cell-free system. In addition, a previous study made in this Laboratory (Simkin & Work, 1957*b*) has suggested that there might be some difference between the incorporation of amino acids into the protein of the microsome fraction of intact cells of guinea-pig liver and the incorporation into the microsome protein of a corresponding cell-free system. A microsome–cell-sap system of the type described by Zamecnik & Keller (1954) was used for these studies, and we reported that some soluble proteinaceous material present in the cell-sap fraction became labelled to a significant extent upon incubation. This finding prompted an investigation into the origin of the soluble protein which becomes labelled in this system. The present paper provides evidence suggesting that the microsome fraction does act as site of origin of the labelled soluble protein, and data are reported relating to factors affecting the passage of radioactivity from microsome to cell-sap fractions. This study forms part of a more general investigation into the significance of amino acid incorporation in cell-free systems, in the course of which it is hoped to discover whether specific soluble proteins are formed in such systems and whether the microsome fraction is the source of origin of such proteins.

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EXPERIMENTAL

Animals. As in earlier studies (Simkin & Work, 1957*b*), young guinea pigs (400–500 g.), which had been starved overnight, were used.

Materials. Reagents were prepared as described by Simkin & Work (1957*b*), except that in some experiments a purified preparation of [^{14}C]chlorella-protein hydrolysate was used (see below). Adenosine triphosphate (ATP) and guanosine triphosphate (GTP) were purchased from the Sigma Chemical Co.

Purification of chlorella-protein hydrolysates. Hydrolysates of [^{14}C]chlorella protein were prepared as previously described. The amino acids were adsorbed on a column of Zeo-Karb 225 (H^+) and eluted with aq. 0·3 M-NH_3. The NH_3 was removed *in vacuo* and the residue dissolved in water. The use of such purified preparations resulted in slightly lower protein specific-activity values being obtained compared with values with chlorella-protein hydrolysates not subjected to this treatment. In other respects the results with both kinds of preparation were very