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# METABOLIC PRODUCTS IN PIGEON TISSUES AFTER FEEDING GLUCOSE

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## SUMMARY

[$^{14}C_6$]Glucose was given orally to pigeons. After 3 h, the state—other than glycogen or fatty acids—in which radioactive carbon was present in the tissues was investigated.

Nearly all the radioactive material could be extracted with 5 % trichloroacetic acid. Most of the label thus extracted was recovered in 10 compounds: glucose; fructose diphosphate, glucose 6-phosphate and fructose 6-phosphate; alanine, arginine, aspartic acid, cysteine, glutamic acid and lactic acid. The distribution of the label over these compounds varied considerably in the tissues examined (liver, heart, breast muscle and the remainder of the pigeon).

It appears that a considerable part of the ingested glucose carbon is still in intermediates after 3 h, and not yet in end-products of metabolism.

## INTRODUCTION

In the course of investigations conducted in this laboratory concerning the influence of thiamine deficiency on glucose metabolism in pigeons it was observed that 3 h after oral administration of uniformly labelled [$^{14}$C]glucose to normal pigeons only about 26 % of the isotope is recovered in $CO_2$, glycogen and fatty acids[1]. Further experiments showed that there was only very little radioactive carbon in liver and muscle proteins, bone carbonate, lipid glycerol and the excrements.

Therefore, most of the $^{14}$C administered as glucose was apparently still present in the body of the pigeon, in unknown compounds. The work reported in this paper was aimed at elucidating the nature of these compounds.

## MATERIALS AND METHODS

### *Treatment of the animals*

The blue-gray pigeons used in these experiments were treated as described previously for normal pigeons[1]. After 10 days on a synthetic diet, introduced into the gizzard through a glass tube, they received 4 g of glucose in 10 ml solution, containing a known amount of uniformly labelled [$^{14}$C]glucose (Radiochemical Centre, Amersham), by the same way. Respiratory $CO_2$ was collected for 3 h in the manner previously described[1], after which the animals were killed by decapitation. Further handling of the body varied with the kind of experiment being performed.

*Isolation and determination of metabolites*

In balance studies, used in seeking the most suitable method for extracting the unknown radioactive material, fractions obtained were ashed by dry combustion in a stream of $O_2$ at 550°. The $CO_2$ produced was trapped in carbonate-free 10 % NaOH and converted into $BaCO_3$ as previously described[1]. The dried $BaCO_3$ was weighed and its radioactivity measured under an end-window Geiger counter. In cases where the percentage of the dose of radioactive glucose present in a tissue extract had to be determined the same procedure was followed. To this end a sample of the solution of glucose administered was also converted into $BaCO_3$.

Glucose was determined with anthrone as described previously[1], in blood after deproteinization according to SOMOGYI[3].

The method eventually adopted for the extraction of the unknown radioactive material is described in the experimental section (p. 57 and Scheme 1).

For the separation and identification of the unknown radioactive compounds we tried several methods, such as columns of various ion-exchangers, with little success. We finally employed a method of paper chromatography developed by CALVIN *et al.*[4,5]. In this method the compounds are first separated by two-dimensional paper chromatography, concentrations of radioactivity are located on the paper, cut out, eluted, concentrated and rechromatographed in one dimension, together with appropriate known compounds. By combining measurements of radioactivity with chemical reactions in the strips the unknown radioactive compounds can be identified.

The two-dimensional descending chromatography was performed on sheets of Whatman No. 1 (46 cm × 49 cm) at 20° using as solvents first phenol–water (72 g:28 ml), 18 h, then a mixture of equal parts of *n*-butanol–water (1246:84, v/v) and propionic acid–water (69:79, v/v), mixed just before use, 7 h*. Radioactivity was located on the sheets with a Scott counter[6] constructed in our laboratory, radioactive areas were cut out and eluted with water in the apparatus of CANNY[7] and the eluates concentrated *in vacuo*. Each concentrated eluate was spotted on a strip of Whatman No. 1 (23 cm × 46 or 49 cm), with a spot of the pure compound suspected to be present on one side, and a mixture of the eluate and the pure compound on the other. Two (descending) chromatograms were always run, one in an alkaline solvent: *n*-propanol–ammonia–water (6:3:1, v/v), the second in an acid solvent: *n*-butanol–acetic acid–water (4:1:5, v/v). Radioactivity was measured with a Baird and Tatlock chromatogram scanner. Reactions used for chemical detection were: WADE AND MORGAN's[8] for phosphate esters, TOENNIES AND KOLB's[9] for amino acids, PARTRIDGE's[10,11] for glucose and spraying with bromocresol green indicator for lactic acid[12]. To produce enough material for this identification by one-dimensional chromatography, 12–24 two-dimensional chromatograms of an extract were made and processed as above.

To determine the distribution of radioactivity over the compounds separated from an extract, an amount of the extract was spotted on paper and the total deposited activity counted. Thereafter a two-dimensional chromatogram was developed and cut into 1.5 cm × 1.5 cm squares; each square was counted in a windowless gas-flow counter (Radiation Counter Laboratories, Skokie, Ill.). The measured

* To obtain reproducible results all the phenol must be removed from the paper before applying the second solvent. Therefore the sheets were hung for 24 h in a well-ventilated compartment and then heated at 80° for another 1.5 h.

radioactivity was mapped on a second two-dimensional chromatogram (identical with the first) showing the compounds after colour reactions, and assigned to each spot to the best of our ability. We then calculated the fraction of the total activity residing in each spot.

## EXPERIMENTS AND RESULTS

### *Extraction of the unknown radioactive material*

In preliminary experiments, conducted to find the method that would extract most of the radioactive carbon remaining in the body of a pigeon 3 h after oral administration of labelled glucose, an animal was used that had received a dose of 50 $\mu$C. The whole body (excluding the feathers) was frozen in liquid air and pulverized. Part of the material was freeze-dried, the remainder was allowed to thaw. Portions of dry and/or wet tissue were extracted with either 5 % trichloroacetic acid solution, methylal ($CH_2$–$(OCH_3)_2$), acetone or light petroleum (b.p. 45–60°). Extraction of the wet tissue with 5 % trichloroacetic acid appeared to be the most promising method.

TABLE I

RECOVERY OF $^{14}C$ AFTER EXTRACTING A COMPLETE PIGEON WITH 5 % TRICHLOROACETIC ACID

Whole animal extracted three times with 900 ml 5 % trichloroacetic acid in Waring Blendor; each trichloroacetic acid extract washed six times with 350 ml ether; remaining fluid = water layer. Ether washings of each trichloroacetic acid extract combined = ether layer. At first the layers did not separate cleanly, therefore the interphase was also examined. Radioactivity measured as $BaCO_3$ after conversion of organic material to $CO_2$ (see METHODS).

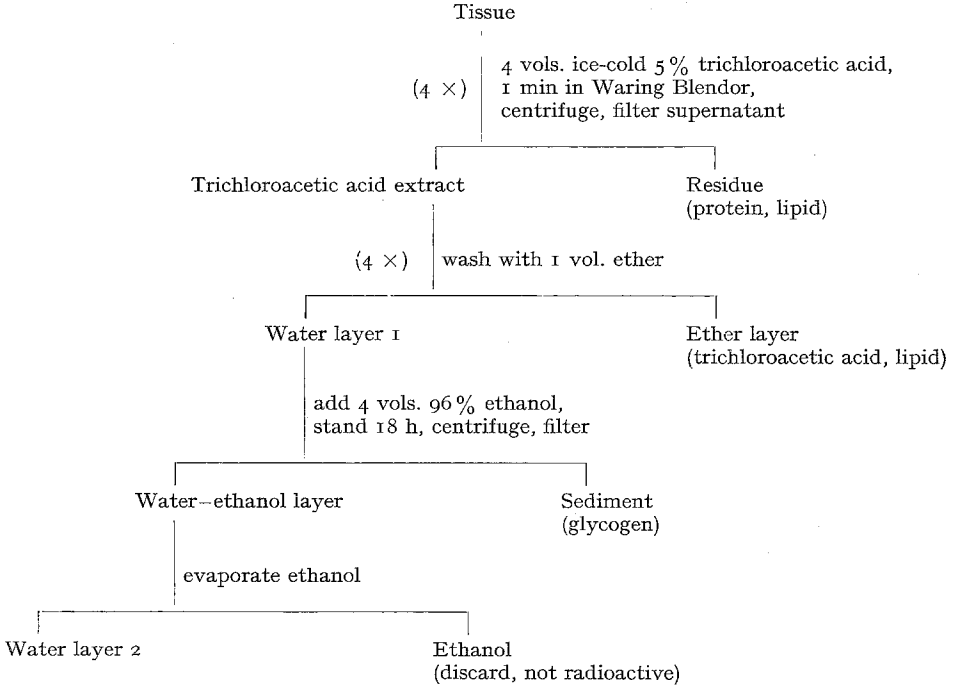
| *Fraction* | $^{14}C$ *(% of dose)* |
|---|---|
| Water layer 1 | 36.0 |
| Interphase 1 | 1.6 |
| Ether layer 1 | 0.1 |
| Water layer 2 | 29.0 |
| Interphase 2 | 0.42 |
| Ether layer 2 | 0.02 |
| Water layer 3 | 7.0 |
| Interphase 3 | 0.02 |
| Ether layer 3 | 0.02 |
| Residue (protein, lipid, bones, etc.) | 13.0 |
| $CO_2$ exhaled | 10.7 |
| Excrements | 0.9 |
| Total | 98.8 |

In a second experiment, therefore, another pigeon was given 20 $\mu$C [$^{14}C_6$]glucose. After 3 h in a metabolism cage the animal was decapitated, its feathers removed, the whole body chopped up and extracted three times in a Waring Blendor at 4° with 5 % trichloroacetic acid. The acid was removed from each extract by repeated shaking with ether and the amount of $^{14}C$ (as $BaCO_3$) determined in each fraction. As Table I shows, most of the isotope was found in the watery residues after extraction, and the recovery of the administered $^{14}C$ was satisfactory (98.8 %).

We finally developed a scheme of extraction, shown in Scheme 1, in which the tissue was extracted 4 times with trichloroacetic acid, and the extracts were pooled before removing the acid with ether. The remaining watery extract was our starting material for the investigation of the unknown radioactive compounds. From it glycogen was first removed by precipitation with ethanol.

*Separation and identification of radioactive compounds in the watery extract*

The procedure of chromatography on paper finally adopted (see METHODS) was first tried out on a combined extract of heart and liver of a pigeon, as this had the highest specific activity (280 counts/min per mg dry weight). The extract was obtained from a pigeon that had been given 100 $\mu$C radioactive glucose. (The breast muscles and the remainder of the animal were also extracted separately according to Scheme 1; the activity of the watery extract was 40 counts/min per mg in both cases.)




Scheme 1. Extraction of radioactive material from a pigeon.

On a two-dimensional chromatogram of the heart–liver extract about 12 concentrations of radioactivity were found. Colour tests applied to several of these chromatograms revealed the presence of phosphate esters, amino acids, other organic acids, and glucose. Maltose could not be detected.

The same extract was next used to examine the possibility of making reproducible two-dimensional chromatograms, as it was obvious that one chromatogram would not give enough material for rechromatography after elution. This indeed proved to be possible. We also confirmed the observation of BENSON *et al.*[4], that a

satisfactory separation is only achieved if the amount of inorganic material applied to the paper with the extract is kept below 300 $\mu$g.

We further made model chromatograms with mixtures of a number of pure compounds that might be present in the extract, to become acquainted with their relative positions on the paper. These were: the amino acids alanine, aspartic acid, arginine, glutamic acid, phenylalanine, leucine and valine; the sugars glucose, fructose diphosphate, glucose 6-phosphate and fructose 6-phosphate, and cysteine·HCl, lactic acid, maltose and 3-phosphoglycerate. The last four compounds were only chromatographed separately, the sugars both separately and in combination, the amino acids in increasing numbers, beginning with alanine and adding one at a time. In the mixtures the positions of the compounds were unchanged relative to their position on the paper when run separately. The mixture of glucose 6-phosphate and fructose 6-phosphate did not separate on the paper.

We made 16 two-dimensional chromatograms of the heart–liver extract, cut out 11 areas of radioactivity from each sheet of paper, eluted the corresponding areas and rechromatographed the eluates in one dimension together with appropriate pure compounds, as described under METHODS. We identified a number of compounds, but owing to the generally low specific activities in only few cases were we able definitely to establish that they were radioactive (alanine, arginine, cysteine, glutamic acid, glucose, glucose 6-phosphate plus fructose 6-phosphate, lactic acid). Glycolic acid was present in fair amount, but was not radioactive.

As the amount of extract that can be chromatographed is limited by its content of inorganic material (see above), we tried to increase the specific activity of the compounds in the tissues by giving a pigeon 500 $\mu$C of labelled glucose, and used this animal for an experiment. Separate extracts were made of heart, liver, breast muscles, blood and the remainder of the animal, because we considered it likely that the distribution of the $^{14}C$ over the unknown metabolites would not be the same in all tissues. These extracts were examined by the method tried out with the heart–liver extract.

Table II shows the compounds identified after the second, one-dimensional,

TABLE II

COMPOUNDS IDENTIFIED BY PAPER CHROMATOGRAPHY IN EXTRACTS OF PIGEON TISSUES

+, present, radioactive; —, present, not radioactive; o, not detected.

| *Compound* | *Heart* | *Liver* | *Breast muscles* | *Remainder* | *Blood* |
|---|---|---|---|---|---|
| Fructose 1,6-diphosphate | + | + | + | o | o |
| Glucose 6-phosphate + + fructose 6-phosphate | + | + | + | o | o |
| 3-Phosphoglycerate | — | — | — | o | o |
| Alanine | + | + | + | + | o |
| Arginine | + | + | + | + | o |
| Aspartic acid | + | + | + | o | o |
| Cysteine | + | + | o | o | o |
| Glutamic acid | + | + | + | o | o |
| Glucose | + | + | + | + | + |
| Lactate | + | + | + | + | o |

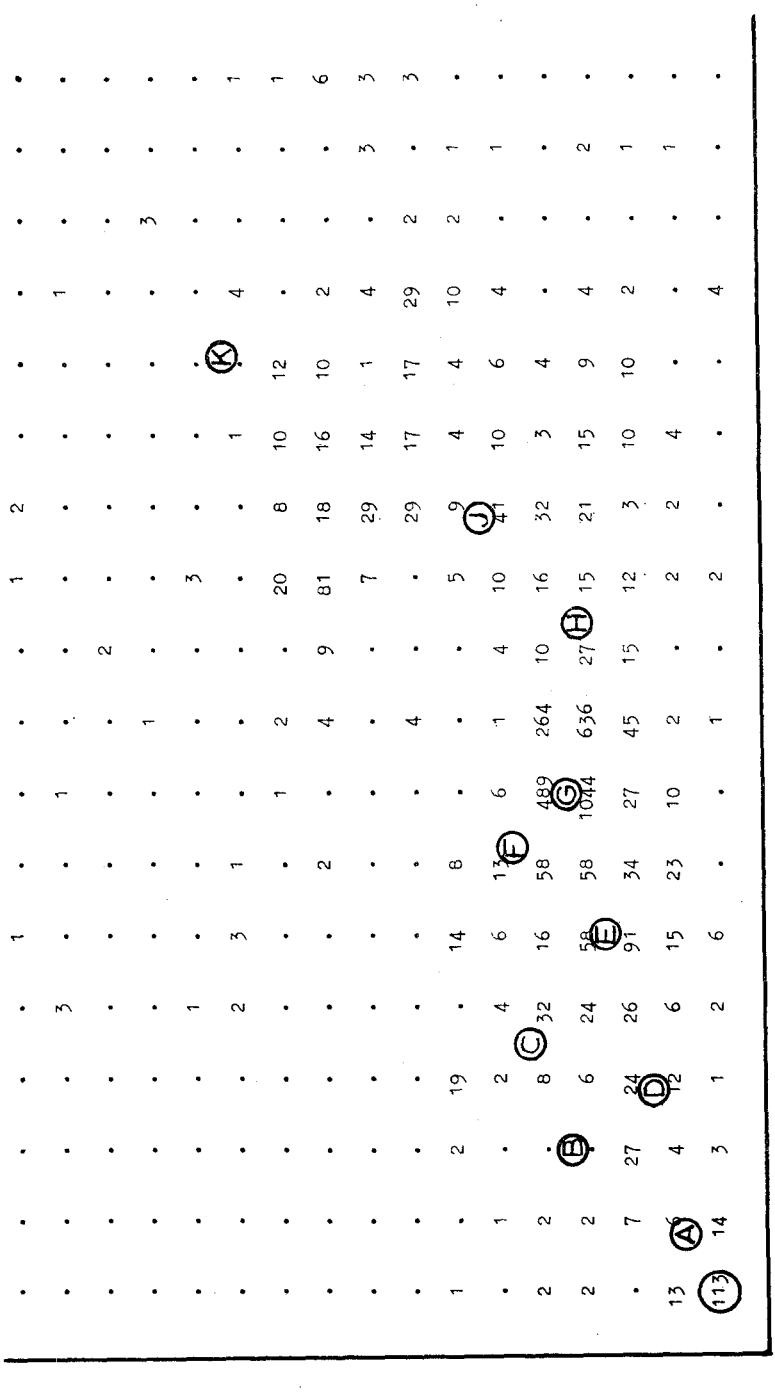



Fig. 1. Distribution of radioactivity in a chromatogram of liver extract. Distance run in first dimension 25.1 cm, in second 24.9 cm. Spotted, 3924 counts/min; recovered, 3514 counts/min. Circles denote centres of spots obtained with pure compounds. A, fructose diphosphate; B, 3-phosphoglycerate; C, aspartic acid; D, glucose 6-phosphate + fructose 6-phosphate; E, cysteine; F, glutamic acid; C, glucose; H, arginine; J, alanine; K, lactic acid.

chromatography of the eluates. Phosphoglycerate, although present in fairly large amount, appeared not to be radioactive.

Fig. 1 shows the distribution of the radioactivity in a two-dimensional chromatogram of liver extract, and Fig. 2 shows the spots revealed by colour reactions on such a chromatogram. The circles marked with letters show the positions of centres of the spots obtained in model experiments with pure compounds. There was some displacement in the case of the biological material, although the relative position of the spots did not change much. Nevertheless this displacement makes the exact location on the chromatogram of the radioactive compounds, present in the extract,

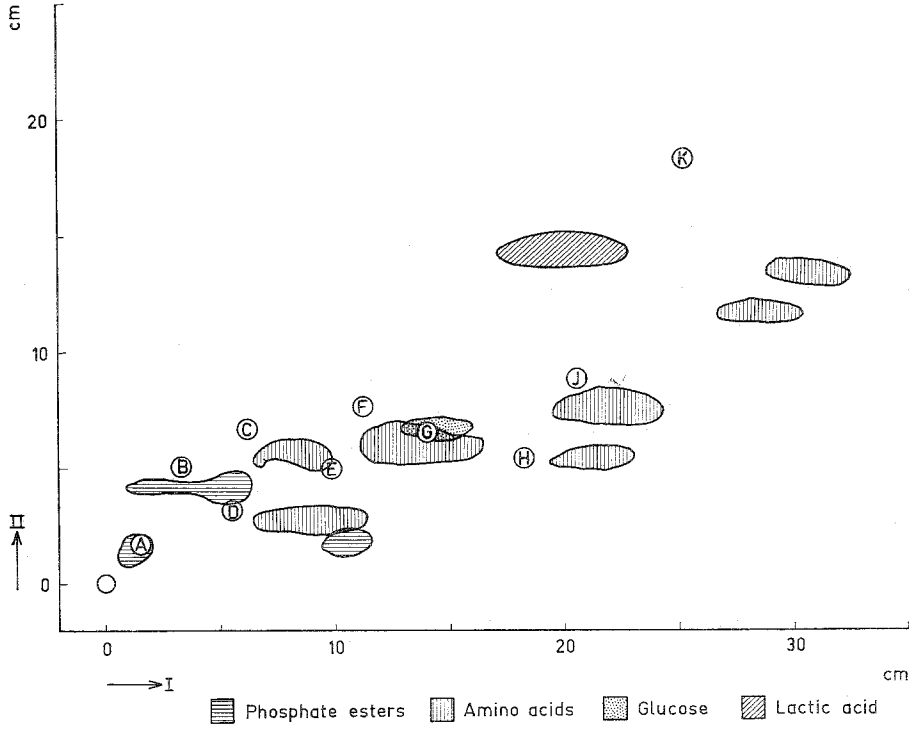



Fig. 2. Chromatogram of liver extract. Reconstruction of 4 separate chromatograms, each sprayed to reveal the presence of amino acids, phosphate esters, glucose and lactic acid respectively. See Fig. 1 for the meaning of circled letters.

a little uncertain. And this we had to know in order to calculate the distribution of the radioactivity over the compounds. Therefore, once the unknown compounds had been identified, we made a series of chromatograms of the extract, each time mixed with a little of one of the pure compounds, and established its precise location by observing the increased intensity of one of the spots.

Table III gives a survey of the distribution of the radioactivity over the compounds in the different extracts. This distribution varied considerably from extract to extract. In all fractions a fair proportion of the $^{14}C$ derived from glucose was present in a number of amino acids, especially in breast muscle tissue. In heart tissue in particular a good deal of the radioactivity was recovered in lactic acid. In blood, glucose was the only radioactive compound found, which is not surprising.

TABLE III

DISTRIBUTION OF RADIOACTIVITY OVER COMPOUNDS IDENTIFIED IN EXTRACTS OF PIGEON TISSUES

Radioactivity of each compound expressed as percentage of the total radioactivity in each extract.

| *Compound* | *Heart* | *Liver* | *Breast muscles* | *Remainder* |
|---|---|---|---|---|
| Fructose 1,6-diphosphate | 5.6 | 4.4 | 8.8 | — |
| Glucose 6-phosphate + + fructose 6-phosphate | 1.2 | 1.5 | 4.5 | — |
| Alanine | 6.2 | 7.0 | 17.6 | 1.6 |
| Arginine | 7.2 | 4.8 | 12.0 | 3.2 |
| Aspartic acid | 2.7 | 1.8 | 1.4 | — |
| Cysteine | 6.0 | 7.7 | — | — |
| Glutamic acid | 4.2 | 7.8 | 4.5 | — |
| Glucose | 35.2 | 56.6 | 20.4 | 53.3 |
| Lactic acid | 22.8 | 6.4 | 17.3 | 15.3 |
| Unaccounted for | 9.9 | 2.0 | 13.5 | 26.6 |

It is striking that so much radioactive carbon is still present as glucose in the extracts, especially in the liver. Further experiments were performed to test the possibility that this is due to blood retained in the tissue after decapitation. Four pigeons that had been given 4 g of unlabelled glucose were used to determine the blood sugar level 3 h later; in two others, treated in the same manner, blood retained in the liver was estimated by spectroscopic determination of haemoglobin; and from three such pigeons liver extracts were prepared according to Scheme 1, in which glucose was determined enzymically[13]. The livers indeed contained a considerable amount of glucose: 107–158 mg. On average the glucose content of the blood was 225 mg per 100 ml at the time of killing, and there was 0.09 mg blood per g of liver. So in the liver of our radioactive pigeon, weighing 8.5 g, only 1.7 mg of glucose could be attributed to the blood in the tissue, and most of it must have been present in the tissue fluid proper.

In all cases some $^{14}C$ cannot be accounted for. This unidentified radioactivity is especially high in the remainder of the pigeon, where only a few compounds could be identified with certainty owing to the low specific activity of the extract. (450 counts/min per 250 $\mu$g inorganic material; *cf.* liver extract, 3500.)

## DISCUSSION

Earlier investigations[1] had shown that, 3 h after oral administration of radioactive glucose to pigeons, most of the label is still present in the tissues, only little of this in the form of glycogen (6.6 %) and fatty acids (6.8 %). This is in contrast to the general opinion prevailing since the work of STETTEN AND BOXER[14] that carbohydrate, not oxidized to $CO_2$ or stored as glycogen, will largely be converted into fat.

While the present investigation was in progress, it came to our notice that DRURY *et al.*[2] had made similar observations: after giving eviscerated rabbits an infusion of radioactive glucose for 8–9 h, they recovered 2.7 % of the dose in glycogen, 0.2 % in fatty acids, and 17.2 % in expired $CO_2$. However we have not found any evidence in the literature that they have pursued this matter.

The work reported in this paper shows that most of the missing $^{14}C$ is found in other compounds, namely: (a) glucose; (b) the hexose phosphates: fructose 1,6-diphosphate, glucose 6-phosphate and fructose 6-phosphate; (c) the amino acids: alanine arginine, aspartic acid, cysteine and glutamic acid; (d) lactic acid. With the exception of glucose and lactic acid, they are all intermediates of various metabolic pathways and not end-products of these pathways.

Somewhat comparable experiments have been performed by SMITH AND MOSES[15], who incubated slices of rat tissues (liver, heart and brain) with uniformly labelled [$^{14}C$]glucose, and found the label in a number of compounds also mostly falling within Groups (a)–(d) mentioned above. So apparently the pattern of metabolism of glucose *in vitro* is similar to that which we have observed *in vivo*.

We were surprised to find so much free glucose in the tissues, although the presence of this compound has also been observed by others: SMITH AND MOSES[16] found labelled glucose after incubating tissues with [2-$^{14}C$]acetate, and MUNTZ AND VANKO[17] reported the presence of 100–200 $\mu$moles of radioactive free glucose in rat livers after intraportal injection of [$^{14}C$]fructose.

It might be supposed that this glucose could have arisen through enzymic breakdown of glycogen in the time elapsing between the death of the animal and the homogenization of the liver in trichloroacetic acid, since MUNRO *et al.*[18] observed that 11 % of the glycogen in a liver is converted into glucose if one waits 5 min instead of 1 before placing the tissue in 30 % KOH. However in our experiments this time interval was not more than 1 min. Nor is it likely that the glucose comes from hydrolysed glucose 1-phosphate, because we find only little of the 6-isomer, which moreover contains very little of the isotope, and we do not expect that one glucose phosphate ester would be very much in excess of the other.

A considerable amount of $^{14}C$ has been found in some amino acids, but in earlier investigations very little radioactivity was encountered in the proteins of liver and breast muscle[1,19]. Evidently there has been only little incorporation of these amino acids into protein in 3 h. The rate of renewal of most proteins seems to be low; indeed MCFARLANE[20] estimates it at 1–2 % per day[20].

To find $^{14}C$ derived from glucose in lactic acid is not surprising, but the amount in the heart extract was unexpectedly large. Perhaps this can be explained by the fact that the heart muscle continues to contract after decapitation, thereby breaking down glucose to lactic acid.

The specific activity of the extract from the remainder of the pigeon was obviously too low to permit a satisfactory examination. However we do not expect that, had it been higher, we would have found in it radioactive compounds different from those encountered in the other tissues: probably we would have found more of the same.

In all extracts some $^{14}C$ could not be accounted for; understandably, this amount increased with decreasing specific activity of the extract (Table III). Possibly we might be able to detect other radioactive metabolites after giving pigeons a very much larger dose of [$^{14}C$]glucose. These would be compounds of very low specific activity, or compounds present in very low concentration. Regarding the first possibility, one could think of 3-phosphoglycerate, that was present in large amount, but in which we could not detect any radioactivity. SMITH AND MOSES[15], whose dose of $^{14}C$ was relatively much higher than ours in their experiments *in vitro*, found the label in 3-phosphoglycerate, but much less than in the hexose phosphates, the

activity of which was very low in our extracts. Regarding the second possibility (compounds present in very low concentration in the tissues) one might expect to find radioactive triose phosphates and intermediates of the citric acid cycle. But the still unknown compounds would contain very little radioactivity compared with the ten we have been able to identify, considering that we did not find more labelled compounds in the liver extract than in that of the heart, although the specific activity of the former was more than 3 times that of the latter. More probably, with a higher dose we would find more radioactivity in the same ten compounds and be left with less unaccounted for.

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