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The Phosphate Esters of Mammalian Skin Maintained on Glucose and Various Deoxyglucoses

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In most living cells the first step in glycolysis is phosphorylation of glucose at C-6, catalysed by the enzyme hexokinase. Studies with hexokinase preparations from yeast and from brain show that compounds closely related to glucose may also be phosphorylated, e.g. 1:5-anhydro-D-glucitol, 2-deoxy-D-glucose, D-glucosone, D-glucosamine and D-mannose (Harpur & Quastel, 1949; Brown, 1951; Johnstone & Mitchell, 1953; Sols & Crane, 1954; Woodward & Hudson, 1955), but 3-deoxy-D-glucose, N-acetyl-D-glucosamine and D-galactose are not phosphorylated (Grant & Long, 1952; Sols & Crane, 1954). Of these compounds 2-deoxyglucose, glucosone and glucosamine are known to interfere with tumour-cell glycolysis (Woodward & Hudson, 1954; Yushok & Batt, 1957). 2-Deoxyglucose is also reported to inhibit the synthesis of influenza virus in chick embryos (Kilbourne, 1959), presumably by functioning as an antimetabolite of glucose. Wick, Drury, Nakada & Wolfe (1957) concluded that the inhibition of glycolysis which occurred when 2-deoxyglucose was given to rabbits was at the phosphoglucosomerase stage of glycolysis. Other than this there seems to be very little published information about the effects of deoxy-sugars on normal mammalian cells and further experiments seemed desirable. Tissue culture affords a convenient approach to this problem and in the experiments to be described slices of mammalian skin were maintained on media containing 1:5-anhydro-D-glucitol (which may be regarded as 1-deoxy-D-glucose), 2-deoxy-D-glucose and 3-deoxy-D-glucose (Fig. 1). D-Glucosone, D-mannose and D-glucosamine were also included since these hexoses differ from glucose at C-2.

The metabolism of the skin cells has been studied by micro-respirometry and by chromato-

graphy and radioautography of the phosphate esters formed during maintenance on radioactive inorganic phosphate. It was first necessary to identify the phosphate esters of skin maintained on glucose and then to examine skin maintained on deoxy-sugars. For this purpose certain phosphate esters

Fig. 1. Structural formulae of deoxyglucoses.

were prepared by the action of yeast hexokinase and used to identify the corresponding [^{32}P]esters produced by skin cells.

EXPERIMENTAL

Materials

1:5-Anhydro-D-glucitol. This was prepared by the reduction of 2:3:4:6-tetra-*O*-acetyl-1-bromo- α -D-glucopyranoside with lithium aluminium hydride (Ness, Fletcher & Hudson, 1950). The product had m.p. 142° and $[\alpha]_D^{20} + 42.4^\circ$.

2-Deoxy-D-glucose. This was prepared from glucose via 3:4:6-tri-*O*-acetyl-D-glucal (Overend, Stacey & Staněk, 1949). The triacetylglucal was recrystallized several times to ensure that the final product was free from glucose. The product had m.p. 148° and $[\alpha]_D^{20} + 46.6^\circ$. No glucose could be detected in a 15% (w/v) solution subjected to paper electrophoresis.

3-Deoxy-D-glucose. This was obtained by hydrolysing 3-deoxy-1:2:5:6-di-*O*-isopropylidene- α -D-glucose (kindly supplied by Dr E. J. Hedgley) in water at 100° with an ion-exchange resin.

D-Glucosone. This was kindly provided by Dr S. Bayne. This substance, which is extremely difficult to purify, was available as a syrup which was freeze-dried to facilitate weighing. Electrophoresis in borate buffer on paper (Foster & Stacey, 1955) and paper chromatography (phenol-water; 4:1) showed that the major constituent M_R 0.95 and R_F 0.74 was accompanied by traces of two other substances M_R values 0.38 and 0.65, R_F values 1.65 and 1.91. (M_R applies to electrophoresis and is the distance migrated by the substance divided by the distance migrated by glucose, the distances being corrected for endosmotic flow. R_F applies to chromatography and is the distance moved by the substance divided by the distance moved by glucose.) No glucose could be detected even when a 15% (w/v) solution was applied to the papers and therefore this sample was considered suitable for metabolic experiments.

N-Acetyl-D-glucosamine. This compound, m.p. 206° (decomp.), was prepared by acetylation of D-glucosamine (Pierce & Kane, 1954).

Phosphate esters. 1:5-Anhydro-D-glucitol, 2-deoxy-D-glucose, D-mannose, D-glucosone and glucosamine were each phosphorylated with yeast hexokinase and adenosine triphosphate (ATP). The resulting phosphate ester was separated by fractional precipitation of the barium salts with ethanol, as described by Brown (1951) for the preparation of glucosamine 6-phosphate. The esters from 1:5-anhydro-D-glucitol and 2-deoxyglucose showed the same R_F value when chromatographed on paper in propan-1-ol-aq. NH_3 soln. as the chemically synthesized esters (Brooks, 1959). The following were also used as reference substances on chromatograms: dipotassium- α -D-glucose 1-phosphate dihydrate ($[\alpha]_D + 78.8^\circ$), prepared from starch by the action of potato phosphorylase, D-glucose 6-phosphate, D-fructose 6-phosphate, D-fructose 1:6-diphosphate and 3-phospho-D-glyceric acid (British Drug Houses Ltd.), adenosine mono-, di- and tri-phosphate (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.) and uridine diphosphoglucose, guanosine di- and tri-phosphate, uridine di- and tri-phosphate and phosphoenolpyruvic acid (Sigma Chemical Co., St Louis, Mo., U.S.A.). Where necessary, barium salts were converted into the corresponding sodium salts before use.

Methods

Tissues and media. Slices of guinea-pig skin were obtained from the ear of the adult animal as described by Cruickshank (1954).

The basal medium was Krebs-Ringer phosphate (Field, 1948) containing 50 μg . of streptomycin sulphate (Glaxo Laboratories Ltd.)/ml. Substrates were prepared at 0.2M in 0.85% NaCl and sterilized by filtration through sintered glass (porosity no. 5; maximum pore size $< 1.0 \mu$).

Respiration studies. The differential microrespirometer described by Cruickshank (1954) was used for respiration studies. This instrument enables the respiration of small quantities of skin to be measured accurately for periods of up to 3 days. Between 8 and 11 mg. of skin floating on 0.45 ml. of Krebs-Ringer phosphate was assigned to each respirometer and the oxygen uptake measured for 1.5 hr. at 37° , the gas phase being air. Substrate (0.05 ml.) was then added by means of a Pasteur pipette, making a 1:10 dilution with the basal medium and giving a final substrate concentration of 0.02M. For experiments with no substrate 0.05 ml. of 0.85% NaCl was added. Respiration measurements were then continued for a further 4–5 hr.; they were discontinued overnight (about 16 hr.) and were recommenced the following day and continued up to 24 hr. after addition of the substrate. All changes in respiratory rate due to substrate were then related to the initial 1.5 hr. control period without substrate.

No measurements of pH changes were made although Cruickshank (1954) quotes pH values between 8.5 and 8.0 during respiration experiments over periods of 24 hr.

Phosphate ester studies. For each experiment about 20 mg. of skin was maintained on Krebs-Ringer phosphate containing 0.15 mc of [^{32}P]orthophosphate in 10 ml. It was found best to distribute the skin among three or four containers each holding 0.45 or 0.9 ml. Substrate solution (or medium only) was added to make a final concentration of 0.02M and the systems were incubated for 24 hr. at 37° . After this time sterility was checked by streaking a loopful of the medium across a blood-agar plate; this was incubated for 1 day. The skin was ground in a hand-operated glass tissue-grinder (A. Gallenkamp and Co. Ltd.) in a minimal quantity of water. Solid material was removed by centrifuging and the protein was precipitated by addition of one drop of trichloroacetic acid (100%, w/v). The precipitate was removed by further centrifuging and the trichloroacetic acid neutralized by addition of aq. NH_3 soln. [sp.gr. 0.880 diluted 1:1 (v/v) with water]. Approximately 5 mg. of ethylenediaminetetra-acetic acid (EDTA) was then added and the resultant solution (about 0.1 ml.) used for chromatography; this was stored at -20° . Extracts from skin contaminated with bacteria or fungi were discarded.

Chromatography. Two-dimensional chromatography based on the method of Hanes & Isherwood (1949) was used. Sheets of Whatman no. 1 filter paper ($18\frac{1}{2} \times 22\frac{1}{2}$ in.) were soaked overnight in 0.2% EDTA adjusted to pH 7.8 with NaOH. They were then washed several times with water and allowed to dry at room temperature.

About 10 spots of skin extract were successively dried on the paper (in certain experiments solutions of reference substances were mixed with the skin extract at this stage), and chromatographed in propan-1-ol-aq. NH_3 soln. (sp.gr. 0.880)-water (60:30:10, by vol.) for 40 hr. The solvent front had run well clear of the paper after this time. The

paper was dried, rotated through 90° and chromatographed in picric acid-2-methylpropan-2-ol-water (4:80:20, w/v/v) for 24 hr. The solvent front was usually about 2 in. from the bottom of the paper.

Phosphate esters were present in amounts too small for detection by chemical means and only inorganic phosphate was detected by spraying. Radioautographs were prepared by attaching the chromatogram to the packet containing a Kodirex X-ray film (14 in. × 17 in.) by means of drawing pins at the origin and two corners. An exposure of 9 days was found adequate for an extract prepared from 20 mg. of skin cultured on media containing about 15 μ C of [32 P]/ml. on commencing the exposure. Weaker extracts were given correspondingly longer exposures calculated from the radioactive decay.

Reference compounds were detected by spraying with a solution containing 5 ml. of 60% (w/w) perchloric acid, 10 ml. of N-HCl, 25 ml. of 4% (w/v) ammonium molybdate and water to 100 ml., and heating at 85° for 7 min. (Hanes & Isherwood, 1949). The paper was then sprayed with 5% (w/v) ascorbic acid and phosphate esters appeared as blue spots (Leuthardt & Testa, 1951). The chromatogram and radioautograph were placed together, the pin-holes being used to locate the two, and the blue area was traced on to the film thus forming a permanent record. If the marker compound was present in the skin extract one of the areas of blackening coincided with the blue coloration.

Because of their resistance to acid hydrolysis some hexose 6-phosphates cannot be readily detected by the molybdate reagent. Such esters were demonstrated by spraying with aniline hydrogen phthalate; this process does not interfere with subsequent detection of other phosphate esters by the molybdate reagent.

RESULTS

Respiration on hexoses and deoxyglucoses

It was found that skin maintained without substrate respired steadily for the first 2 hr., presumably utilizing endogenous substrate, after which time the respiration rate fell gradually. If the 0-1.5 hr. period is taken as 100%, the next 2-hr. period shows a decrease of about 15% and the 22-24 hr. period a decrease of about 65%. If glucose (0.02M) was added after 1.5 hr. the respiration rate was still at the initial rate 4 hr. later and decreased by only 18% after 24 hr. These findings are in agreement with the observations made by Cruickshank, Trotter & Cooper (1957).

It was found that mannose (0.02M) and galactose (0.02M) were able to support the respiration of skin to the same extent as glucose (Table 1). Though the percentage of the original value for skin maintained in galactose is lower than that for glucose (77 and 84% respectively) the difference is not statistically significant for the number of experiments carried out.

Glucosamine (0.02M) also supported skin respiration, but not as efficiently as glucose, respiratory survival being 56%, a significantly lower amount. Glucosone and 1:5-anhydro-D-glucitol could also support skin respiration to a similar extent (about 50% survival after 24 hr.). However, these two

Table 1. *Respiration of guinea-pig skin on various substrates*

Respiratory rates are given $\pm$ S.E.M.

Substrate (concn. 0.02M)	No. of experiments	Uptake of oxygen				
		0-1½ hr. before substrate was added (μ l./mg. wet wt./hr.)	0-2 hr. after substrate was added (μ l./mg. wet wt./hr.)	Percentage of initial rate	22-24 hr. after substrate was added (μ l./mg. wet wt./hr.)	Percentage of initial rate
None	9	0.70 $\pm$ 0.02	0.60 $\pm$ 0.02	86	0.23 $\pm$ 0.01	33
D-Glucose	11	0.69 $\pm$ 0.03	0.69 $\pm$ 0.04	100	0.58 $\pm$ 0.03	84
1:5-Anhydro-D-glucitol	4	0.63 $\pm$ 0.01	0.71 $\pm$ 0.02	113	0.35 $\pm$ 0.05	56
1:5-Anhydro-D-glucitol + D-glucose	4	0.68 $\pm$ 0.06	0.73 $\pm$ 0.06	107	0.54 $\pm$ 0.05	79*
2-Deoxy-D-glucose	6	0.69 $\pm$ 0.02	0.56 $\pm$ 0.03	81	0.21 $\pm$ 0.03	30†
2-Deoxy-D-glucose + D-glucose	4	0.66 $\pm$ 0.08	0.58 $\pm$ 0.09	88	0.31 $\pm$ 0.05	47
3-Deoxy-D-glucose	5	0.71 $\pm$ 0.02	0.62 $\pm$ 0.02	87	0.25 $\pm$ 0.03	35†
3-Deoxy-D-glucose + D-glucose	5	0.63 $\pm$ 0.03	0.62 $\pm$ 0.03	98	0.54 $\pm$ 0.04	86*
D-Glucosone	5	0.66 $\pm$ 0.02	0.79 $\pm$ 0.07	120	0.30 $\pm$ 0.03	45
D-Glucosone + D-glucose	5	0.61 $\pm$ 0.02	0.69 $\pm$ 0.05	113	0.50 $\pm$ 0.02	82*
D-Glucosamine	8	0.72 $\pm$ 0.03	0.71 $\pm$ 0.04	99	0.40 $\pm$ 0.06	56
D-Glucosamine + D-glucose	3	0.68 $\pm$ 0.07	0.66 $\pm$ 0.07	97	0.57 $\pm$ 0.06	84*
N-Acetyl-D-glucosamine	6	0.65 $\pm$ 0.01	0.65 $\pm$ 0.02	100	0.22 $\pm$ 0.04	34†
N-Acetyl-D-glucosamine + D-glucose	3	0.65 $\pm$ 0.05	0.62 $\pm$ 0.08	95	0.51 $\pm$ 0.06	78*
D-Mannose	11	0.71 $\pm$ 0.03	0.70 $\pm$ 0.04	99	0.57 $\pm$ 0.03	80*
D-Galactose	8	0.68 $\pm$ 0.03	0.69 $\pm$ 0.04	102	0.52 $\pm$ 0.06	76*
D-Galactose + 2-deoxy-D-glucose	3	0.70 $\pm$ 0.02	0.69 $\pm$ 0.02	99	0.57 $\pm$ 0.02	81*

* Not significantly different from glucose.

† Not significantly different from 0.85% NaCl.

substances differed markedly from all the others tested by causing a significant initial increase in the respiratory rate. This increase was about 15% above the original value and persisted for about 3 hr., after which time the rate gradually fell.

The respiration of skin maintained in 2-deoxyglucose, 3-deoxyglucose and *N*-acetylglucosamine was similar to that of skin maintained without substrate; apparently they could not act as an energy source nor were they toxic. *N*-Acetylglucosamine, however, supported respiration for a period immediately after addition; this may perhaps be due to traces of acetate as an impurity which the skin could utilize (Cruickshank *et al.* 1957).

Further experiments with equimolar glucose and 1:5-anhydro-*D*-glucitol, 2-deoxyglucose, 3-deoxyglucose, *N*-acetylglucosamine and glucosone were carried out to see whether these substances were inert or whether they interfered with respiration on glucose. The results are included in Table 1, from which it can be seen that 3-deoxyglucose and *N*-acetylglucosamine apparently did not interfere with glycolysis. 2-Deoxyglucose markedly interfered with glucose utilization, respiratory survival being only 45% compared with 84% for skin maintained in glucose alone. Little respiratory suppression (relative to glucose media) was caused by 1:5-anhydro-*D*-glucitol and *D*-glucosone but interference with normal glucose metabolism may be inferred from the fact that although stimulation of respiration still occurred on mixed substrates it was less than that produced by 1:5-anhydro-*D*-glucitol and *D*-glucosone alone.

2-Deoxyglucose did not interfere with the normal metabolism of galactose.

Phosphate esters formed by skin

In initial experiments five extracts prepared from guinea-pig skin maintained on 0.02M-glucose were chromatographed as described earlier. The radioautographs on development showed a number of spots; a tracing of one of these is shown in Fig. 2. A large dense spot always appeared (no. 5); this was shown to be inorganic phosphate. With inorganic phosphate as a reference spot, R_f values were recorded for the two directions of chromatography. The reproducibility of the pattern of these spots between different experiments was very good, though the identification of spots never depended upon this reproducibility. Fig. 3 shows a composite diagram constructed from the five chromatograms. The esters found were numbered arbitrarily. Twenty-two esters were found consistently on each chromatogram and a further six occurred on some chromatograms (these six are denoted by circles in Fig. 3).

Three experiments were carried out with human skin (surplus to grafting operations); the pattern and number of phosphate esters detected appeared to be identical with those found in guinea-pig skin. The positions of the majority of the esters from human skin were well within the limits shown in Fig. 3.

Fig. 2. Phosphate esters from guinea-pig skin maintained on a medium containing [32 P]orthophosphate and glucose. An extract of the skin was subjected to chromatography in two dimensions. The sketch illustrates the radioautograph of a typical chromatogram. See Table 2 for a key to the identified spots.

Fig. 3. Diagram showing the position on chromatograms of phosphate esters from mammalian skin. Crosses denote the means and rectangles the limits of R_f values in five experiments. Circles denote the position of esters not detected on every chromatogram. See Table 2 for a key to the identified spots.

Further experiments were made with guinea-pig skin extracts to identify as many of these phosphate esters as possible. This was done by mixing phosphate esters at 0.02M with the skin extract as markers. After chromatography the marker was detected by a spray reagent. The coincidence of the colour with the radioautograph image was taken as evidence that the marker substance was present in the skin extract. Usually the coloured spot was larger than the image owing to the relative sensitivity of the two methods; only for inorganic phosphate was the image larger than the blue spot. Distortions of the spot occasionally occurred, e.g. a crescent shape occurred with ATP, but these were accepted where there was reasonable symmetry of image and blue spot.

Usually two separate experiments were made with each marker. Altogether 14 of the regularly occurring skin phosphate esters were identified in this way; these are listed in Table 2 under the heading 'Skin maintained on glucose'. In addition to the compounds listed there was some reason to believe that spot no. 1 was uridine triphosphate, spot no. 2 was uridine monophosphate and spot no. 29 was guanosine triphosphate, but owing to impurities in the reference samples of nucleotides these identifications remain uncertain.

Experiments were made to study the effect of varying the substrate on these phosphate esters. A series of extracts were made from guinea-pig skin maintained on the following substrates at 0.02M: mannose, 1:5-anhydro-D-glucitol, 2-deoxyglucose, 3-deoxyglucose, glucosone, glucosamine, *N*-acetylglucosamine and also without substrate. The radioautographs from skin maintained without substrate, on *N*-acetylglucosamine and 3-deoxyglucose were each similar to those from glucose cultures except that the intensity of the spots was greatly reduced and some of the nucleotides were missing.

Skin maintained on mannose produced chromatograms indistinguishable from chromatograms of skin maintained on glucose.

Chromatograms from skin maintained on other substrates (1:5-anhydro-D-glucitol, 2-deoxyglucose, glucosone and glucosamine) each showed several phosphate esters not normally present (see Table 2, under the heading 'Skin maintained on various substrates'). With 2-deoxyglucose most of the 'normal' spots were eliminated. The intensity of the spots from skin maintained on 1:5-anhydro-D-glucitol, glucosone and glucosamine was reduced compared with those from glucose medium but it was greater than spots from systems without substrate.

Table 2. *Phosphate esters in mammalian skin cells*

Esters were separated by two-dimensional chromatography on paper and provisionally identified by superimposition of the radioautograph image of the [³²P]ester formed in the skin cells with the colour reaction of an authentic sample of the ester added to the skin extract. The table is based on 41 chromatograms and *R_f* values are listed correspondingly; for example, spot no. 20 showed *R_f* 1.25 in propanol-aq. NH₃ soln. (solvent 1) and 0.79 in picric acid-aq. 2-methylpropan-2-ol (solvent 2) on the same chromatogram. *R_f* is the ratio of the distance moved by the phosphate ester to the distance moved by orthophosphate.

Spot no.	Ester	R_f	
		Solvent 1	Solvent 2
Skin maintained on glucose			
5	Orthophosphate	1.00	1.00
6	Fructose 6-phosphate	1.19, 1.26	0.64, 0.68
7	3-Phospho-D-glyceric acid	1.05, 1.16	0.87, 0.88
9b	Uridine diphosphoglucose	1.29, 1.27	0.16, 0.19
10	Uridine diphosphate	0.77, 0.78	0.35, 0.40
11	Adenosine monophosphate	0.95, 1.01, 0.89	0.33, 0.41, 0.33
12	Mannose 6-phosphate	1.08	0.54
13	Glucose 6-phosphate	1.05, 1.09	0.46, 0.41
14	Adenosine diphosphate	0.95, 0.94, 0.89	0.22, 0.25, 0.21
16	Guanosine diphosphate	0.38	0.24
18	Phosphoenolpyruvic acid	1.13, 1.16	1.16, 1.09
19	Adenosine triphosphate	0.71, 0.66, 0.67	0.15, 0.12, 0.06
23	Fructose 1:6-diphosphate	0.40, 0.46	0.68, 0.78
24	Glucose 1-phosphate	1.28, 1.35, 1.33	0.51, 0.51, 0.41
25	Pyrophosphate	0.49, 0.52, 0.56	0.42, 0.45, 0.47
Skin maintained on various substrates*			
20	2-Deoxyglucose 6-phosphate	1.25, 1.34	0.79, 0.77
21	Glucosone 6-phosphate	1.25, 1.36	0.57, 0.59
31	1:5-Anhydro-D-glucitol-6-phosphate	1.31, 1.34	0.77, 0.70
36	Glucosamine 6-phosphate	1.32, 1.38	0.54, 0.57

* The substrates were the unesterified sugar derivatives corresponding to the esters shown.

It was shown that in each case the 'new' esters were distinct from the 'normal' esters of the glucose medium by mixing each extract in turn with a glucose-medium extract and chromatographing the mixture. It was possible to identify one of the new esters from each substrate by mixing a solution of the C-6 phosphate of that substrate into the appropriate skin extract and following the procedure described earlier for ester identification. The R_f values of these compounds have been included in Table 2 under the heading 'Skin maintained on various substrates'.

DISCUSSION

Skin maintained on glucose for 24 hr. shows a 15% decrease in respiration rate after this time, whereas that of skin without substrate decreases by about 65%. These values correspond with those obtained by Cruickshank *et al.* (1957) from similar experiments. The phosphate esters detected in skin maintained on glucose included the majority of the glycolysis-cycle intermediates and certain nucleotides, notably derivatives of adenosine, guanosine and uridine. A similar pattern of esters was obtained from skin without substrate but the intensity of the radioautograph spots was much reduced and many of the nucleotides could not be demonstrated. This indicates that glycolysis was much reduced and the difference between the respiratory rates of skin maintained on glucose and maintained without substrate is consistent with this.

The finding that 3-deoxyglucose and *N*-acetylglucosamine neither act as substrates for skin nor interfere with its respiration on glucose suggested that the pattern of skin phosphate esters would also be unaffected. Chromatography showed that this was indeed the case. This observation is consistent with the inability of hexokinase to act upon *N*-acetylglucosamine (Grant & Long, 1952) and the failure of 3-deoxyglucose to interfere with glycolysis (Woodward, Cramer & Hudson, 1953).

In contrast, 1:5-anhydro-D-glucitol, glucosamine and glucosone act as energy sources for skin metabolism but these substances are not so effective as glucose (Table 1). The respiratory stimulation caused on addition of glucosone and 1:5-anhydro-D-glucitol is interesting; certain bacterial toxins produce a similar effect (Lawrence, 1959) but the cause is unknown. The three substrates, 1:5-anhydro-D-glucitol, glucosamine and glucosone, when mixed with glucose do not inhibit skin respiration as compared with that of cultures on glucose alone. The respiratory stimulation caused by 1:5-anhydro-D-glucitol and glucosone which still occurs in the presence of glucose, though to a less extent, implies an interference with skin metabolism though the effect cannot be precisely de-

fined. The fact that 1:5-anhydro-D-glucitol and glucosone are phosphorylated by hexokinase suggests that there is an interference with glucose phosphorylation. Similarly, glucosamine 6-phosphate was found in extracts from skin maintained on a mixture of glucose and glucosamine. It is known that glucosone and glucosamine inhibit glucose breakdown by tumour cells (Yushok & Batt, 1957).

In skin maintained on glucosone and 1:5-anhydro-D-glucitol four new esters were found: two for each substrate (glucosone nos. 21 and 22; 1:5-anhydro-D-glucitol nos. 31 and 32). Esters nos. 21 and 31 were identified as glucosone 6-phosphate and 1:5-anhydro-D-glucitol 6-phosphate respectively. The intensity of radioactivity of these esters was not strong enough to suggest that they accumulate in skin. The identity of the other two is unknown but it is possible that no. 22 could be glucosone 1-phosphate formed by the action of phosphoglucomutase, which is known to convert phosphoglucomutase, which is known to convert glucosamine 6-phosphate into glucosamine 1-phosphate (Brown, 1953; Distler, Merrick & Roseman, 1958). Clearly 1:5-anhydro-D-glucitol 6-phosphate cannot be affected by phosphoglucomutase and in this case the second ester may be some further intermediate in its breakdown by skin. Chemically it is difficult to visualize how 1:5-anhydro-D-glucitol can be metabolized further than the 6-phosphate. Studies with ^{14}C might throw more light on this problem. The presence of these 6-phosphates is in agreement with previous work; it is known that all are phosphorylated by brain hexokinase in the presence of ATP (Harpur & Quastel, 1949; Sols & Crane, 1954).

Mannose is also known to be phosphorylated by hexokinase, and since chemically it is so closely related to glucose it is not surprising to find that skin can utilize it equally well. The presence of mannose 6-phosphate in normal skin maintained on glucose is noteworthy but certain polysaccharides of connective tissue such as keratosulphate are known to contain mannose (Meyer, Davidson, Linker & Hoffman, 1956), so the presence of its 6-phosphate is perhaps to be expected.

Respiration studies made with 2-deoxyglucose as substrate indicate that it does not affect skin but when mixed with glucose considerable inhibition of glucose metabolism occurs (Table 1). This is in agreement with observations made by Yushok & Batt (1957) and Woodward & Hudson (1954). Chromatography showed the presence of two phosphate esters (nos. 4 and 20) which do not normally occur in skin. These two esters showed a greater radioactivity than did the esters formed by glucosone, the esters of glucosamine and 1:5-anhydro-D-glucitol and the esters found normally, which suggests that they had accumulated in the skin

cells. No. 20 was shown to be 2-deoxy-D-glucose 6-phosphate, which is reported to accumulate in the cells of the rat diaphragm when these are cultured on 2-deoxyglucose (Kipnis & Cori, 1959). The identity of the second ester (no. 4) is unknown.

Galactose supported skin respiration to the same extent as glucose, although hexokinase does not act on galactose under normal conditions (Sols & Crane, 1954) and it seems likely that it is converted into galactose 1-phosphate by galactokinase, an enzyme previously detected in yeasts (Caputto, Leloir, Trucco, Cardini & Paladini, 1949). So far no chromatograms have been prepared from skin maintained on galactose to see whether this is likely, but an experiment in which skin was maintained on equimolar galactose and 2-deoxyglucose showed that 2-deoxyglucose had no effect on the respiration of skin under these conditions. This suggests that an enzyme other than hexokinase is responsible for the utilization of galactose by skin.

The combination of respiration measurements with an examination of phosphate esters in skin provides a useful biochemical approach. Further use of the method should provide information about the metabolism of other sugars by skin and may also have application in determining the site of action of toxic materials both synthetic and microbial in origin.

SUMMARY

1. The phosphate esters of skin have been examined in conjunction with measurements of respiratory activity.

2. Skin maintained on glucose regularly produced detectable amounts of 22 phosphate esters. From time to time six others were observed.

3. Fourteen of the regularly occurring compounds were identified chromatographically and the majority were intermediates in normal glycolysis or were nucleotides.

4. Respiration studies made with other substrates showed that galactose and mannose were as effective as glucose in supporting skin respiration, but *N*-acetylglucosamine, 2-deoxy-D-glucose and 3-deoxy-D-glucose did not support respiration. 1:5-Anhydro-D-glucitol, glucosone and glucosamine supported skin respiration to some extent but were not as efficient as glucose. When mixed with glucose the following inhibited normal glucose

metabolism: 1:5-anhydro-D-glucitol, 2-deoxyglucose, glucosone and glucosamine.

5. Hexose 6-phosphates not normally present in skin were formed from 1:5-anhydro-D-glucitol, 2-deoxy-D-glucose, D-glucosone and glucosamine; of these 2-deoxy-D-glucose 6-phosphate appeared to accumulate in the skin cells. No new phosphate esters were produced from 3-deoxy-D-glucose or from *N*-acetylglucosamine.

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