The effects of starvation and re-feeding on glycogen metabolism in mouse tail skin

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Although the glycogen content of mouse tail skin was decreased during starvation and was restored on re-feeding, the proportion of glycogen synthase in the I form remained constant throughout at about 10% of the total. During the phase of net glycogen synthesis 1.5 h after access to food was restored, the concentration of UDP-glucose was markedly increased and the proportion of phosphorylase in the *a* form was significantly decreased.

The only known function of glycogen is to act as an energy store, and the biochemical mechanisms that regulate its utilization are determined by the special functions of the organ in which it is synthesized. Thus in liver the physiological purpose of the mechanisms controlling glycogen metabolism is to regulate blood glucose, and in muscle it is to provide energy for contraction (Hers, 1976; Cohen, 1980). In skin the functions of glycogen are less well understood, but there is much evidence that epidermal glycogen metabolism is in some way related to keratinocyte growth and differentiation, and in support of this view it has been shown that glycogen accumulates in response to a variety of stimuli, which induce epidermal hyperplasia (Lobitz *et al.*, 1962; Ohkawara *et al.*, 1972; Peters & White, 1978), and that it is present in increased amounts in regenerating epithelium (Im & Hoopes, 1970; Williams, 1972) and in hyperproliferative lesions such as psoriasis (Halprin & Ohkawara, 1966; Halprin *et al.*, 1973). These findings suggest that the control of glycogen metabolism in skin may have distinctive features and, since regulatory mechanisms that operate *in vivo* are most readily investigated under conditions in which the pathway flux is altered, the effects of starvation and re-feeding on glycogen metabolism in mouse tail skin have been investigated.

Experimental

Animals

The experiments were performed on male Balb/c mice, 6–8 weeks old, which were allowed food and

water *ad lib* except when otherwise stated. The mice were killed by cervical dislocation; the tail skin was stripped off in about 30 s and then immediately placed in liquid N₂ and powdered in a pestle and mortar.

Extraction and determination of skin glycogen and glucose

The powdered skin was homogenized in 10 vol. of ice-cold 0.3 M-trichloroacetic acid with a Polytron homogenizer (Kinematica, Lucerne, Switzerland) set at position 7 for three bursts each of 10 s. The homogenate was incubated at 70°C for 2 h, cooled and then centrifuged at 20 000 *g*_{av} for 15 min, and the supernatant used for the determination of glycogen and glucose. All glassware was cleaned in hot conc. H₂SO₄/HNO₃ (3:1, v/v). It was found in preliminary experiments that skin extracts contained 1–2 nmol per mg wet wt. of substance(s) that reduced oxidized 2,2'-azinobis-(3-ethylbenzthiazolinesulphonate) ('ABTS'), but that these substance(s) were destroyed by a few seconds' exposure to 0.1 M-NaOH at room temperature. In consequence, 0.1 vol. of 4 M-NaOH was added to skin extracts prepared as described above and an appropriate volume of the alkaline extract (usually 50–200 µl) was then added to stoppered glass tubes (5 ml capacity) containing 0.2 ml of 1 M-sodium acetate buffer, pH 5.0, 0.02 ml of peroxidase (5 units; 0.25 mg/ml), 0.02 ml of 2,2'-azinobis-(3-ethylbenzthiazolinesulphonate) (25 mg/ml) and 0.76 ml of water. Then 50 µl (50 units) of glucose oxidase (Sigma Type V, from which thimerosal had been removed by passage down a column of Bio-Gel P20) was added and the mixture incubated at 40°C for 45 min. *A*₄₂₀ was then determined and the glucose

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content of the extract calculated. Then 5 μ l (0.7 unit) of amyloglucosidase was added and, after a further 30 min incubation at 40°C, the increment in A_{420} was determined and the glycogen content of the extract calculated. In all experiments standard solutions of glucose and glycogen were assayed, tubes to determine the changes in A_{420} caused by enzyme addition were included in the assay, and the efficacy of the alkali-pretreatment step was tested by showing that oxidized 2,2'-azinobis-(3-ethylbenz-thiazolinesulphonate) remained stable in the presence of skin extract.

Determination of plasma glucose

Blood was collected in heparinized tubes and cells were removed by centrifugation for 2 min at 10000 g_{av} . in a Beckman Microfuge. A sample of the plasma was deproteinized with 0.3 M-trichloroacetic acid (final concn.) and, after centrifugation for 2 min at 10000 g_{av} . to remove precipitated protein, glucose was determined as described above.

Extraction of metabolic intermediates

Frozen skin obtained as described above was powdered under liquid N_2 and homogenized in 30 vol. of ice-cold 0.6 M-HClO₄. The homogenate was centrifuged at 20000 g_{av} . for 30 min at 4°C and the supernatant titrated to pH 6.5–7.0 with 5 M-KOH. The neutralized extract was left on ice for 1 h, centrifuged at 20000 g for 30 min at 4°C and the supernatant was stored at –80°C and used within a few days.

Determination of metabolites

The assays were dependent on the formation of NAD(P)H, which was then measured with the combined NAD(P)H:FMN oxidoreductase and luciferase preparation from *Photobacterium fischeri* (Sigma Chemical Corp., St. Louis, MO, U.S.A.) as described by Campbell & Simpson (1979), and light emission was measured in an LKB Wallac scintillation counter with the coincidence circuit switched off. All assays were performed in triplicate in plastic tubes of 5 ml capacity, selected for low background light emission, and metabolite concentrations were calculated from the light emitted by 50 pmol of each metabolite added as internal standard to control tubes containing tissue extract. In all cases it was shown that NAD(P)H generation was stoichiometric within experimental error, and that light emission from NAD(P)H was linear with concentration over the range 10–100 pmol.

The procedures used to generate NAD(P)H from individual metabolites were as follows.

UDP-glucose. The method of Keppler & Decker (1974) was used. It was found that commercially

available preparations of UDP-glucose dehydrogenase gave an unacceptably high value of light emission, but that the enzyme prepared from bovine liver by the method of Oliver (1961) was suitable.

Glucose 6-phosphate. The method of Lang & Michal (1974), using NAD-dependent glucose 6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (Sigma), was used.

Glucose 1-phosphate and fructose 6-phosphate. Because the concentration of glucose 6-phosphate in skin extracts was relatively high, it was first oxidized as described above and the NADH formed was then destroyed by adding 0.05 vol of 5 M-HCl, and after 5 min at room temperature the reaction mixture was titrated to pH 7.8 with 5 M-NaOH. Glucose 1-phosphate was converted into glucose 6-phosphate by using phosphoglucomutase (Bergmeyer & Michal, 1974), and glucose 6-phosphate was then determined as described above. Similarly fructose 6-phosphate was determined by conversion into glucose 6-phosphate by using phosphoglucoisomerase (Lang & Michal, 1974).

Fructose 1,6-bisphosphate. The method of Michal & Beutler (1974) was used, except that, to minimize quenching, the concentration of NaAsO₄ was decreased to 0.3 mM.

Determination of glycogen synthase and phosphorylase activities

Skin powder was homogenized in 5 vol. of ice-cold 20 mM-Tris/HCl, pH 7.0, containing 0.1 M-NaF and 0.02 M-EDTA by using a Polytron homogenizer set at position 7 for three bursts each of 5 s. The homogenate was centrifuged for 10 min at 25000 g_{av} . at 4°C and the supernatant was used for enzyme assay. Control experiments showed that under these conditions the extraction of both enzymes was optimal and interconversions did not occur. Glycogen synthase I + D activities were assayed by the method of Thomas *et al.* (1968) by incubation for 30 min at 30°C in 75 mM-Tris/HCl (pH 7.6)/5 mM-UDP-glucose containing UDP-[U-¹⁴C]glucose, 5% (w/v) re-purified oyster glycogen and 5 mM-glucose 6-phosphate; for the determination of glycogen synthase I activity, glucose 6-phosphate was omitted. Phosphorylase *a* and *b* activities were measured by the method of Stalmans & Hers (1975) by incubation for 30 min at 30°C in the presence of 25 mM-4-morpholine-ethanesulphonic acid, pH 6.6, 50 mM-glucose 1-phosphate (pH 6.6) containing [U-¹⁴C]glucose 1-phosphate, 1% re-purified oyster glycogen and 1.5 mM-AMP; for measurement of phosphorylase *a* activity, AMP was omitted. In all assays it was shown that the reaction velocity was linear with time throughout the period of incubation, and that enzyme activity was linearly related to the volume of tissue extract.

Results and discussion

Extraction and determination of skin glucose and glycogen

Fig. 1 shows that only about one-third of the total glycogen in mouse tail skin was extracted by homogenization in ice-cold 0.3 M-trichloroacetic acid and that maximum extraction was obtained after incubating the homogenate for 2 h at 70°C. These findings are consistent with previous studies on other tissues (Willstatter & Rhodewald, 1934; Kemp & Kits van Heijningen, 1954). Fig. 1 also shows that the tissue glucose remained constant within experimental error throughout the period of incubation, and these results indicate that during the extraction procedure skin glycogen was not degraded to glucose. However, when a solution of glycogen was incubated for 1 h in 0.3 M-trichloroacetic acid at 70°C, although glycogen degradation was not detected, glucose equivalent to about 3% of the glycogen initially present was released (results not shown). It is possible therefore that the procedure described slightly underestimated tissue glycogen and that tissue glucose was correspondingly over-

estimated to the same extent. On the other hand, it was shown that, whereas the glycogen content of a sample of frozen skin powder extracted by the procedure described was 1.88 $\mu\text{mol/g}$, the value obtained by extracting another portion of the same sample of skin powder with hot 30% (w/v) KOH, followed by ethanol precipitation (van Handel, 1965), was 1.73 $\mu\text{mol/g}$. It is apparent that the estimates of skin glycogen obtained by these two procedures are in reasonable agreement, but the extraction with 0.3 M-trichloroacetic acid at 70°C has the advantage of sensitivity and simplicity, and furthermore the extract can be used not only for the determination of tissue glucose but also, if necessary, for the determination of DNA by the method of Burton (1968).

Fig. 1. Effect of incubation at 70°C on the extraction of glucose and glycogen from mouse tail skin homogenized in 0.3 M-trichloroacetic acid

Powdered mouse tail skin was homogenized in ice-cold 0.3 M-trichloroacetic acid as described in the Experimental section. The zero-time sample was then removed and the remaining homogenate incubated at 70°C. Samples were removed at the time indicated, and after centrifugation at 20000 g_{av} for 15 min the supernatant was used for the determination of glucose (■) and glycogen (●).

Fig. 2. Effect of 24 h starvation and re-feeding on the blood glucose concentration and on the glucose and glycogen contents of mouse tail skin

Blood glucose (▲) and the glucose (○) and glycogen (●) contents of mouse tail skin were determined as described in the Experimental section. The values obtained from starved mice are the means for eight separate determinations, and with re-fed mice tissue from four mice were pooled for analysis. The error bars show S.E.M.

Table 1. *Effect of starvation for 24 h, followed by re-feeding for 1.5 h, on the concentrations of metabolites and on the activities of glycogen synthase I and D and phosphorylase a and b in mouse tail skin*

The values are means $\pm$ S.E.M. for at least five separate determinations, except for the determinations of glucose and glycogen after re-feeding, when the tail skin from eight mice was pooled for analysis.

	Content ($\mu\text{mol/g}$ wet wt. of skin)		
	Control	Starved	Re-fed
Glucose	2.93 ± 0.06	1.88 ± 0.07	3.6
Glycogen	2.11 ± 0.07	0.77 ± 0.19	1.2
UDP-glucose	0.073 ± 0.008	0.072 ± 0.003	0.113 ± 0.007
Glucose 1-phosphate	0.015 ± 0.002	0.022 ± 0.007	0.023 ± 0.005
Glucose 6-phosphate	0.048 ± 0.002	0.095 ± 0.007	0.084 ± 0.006
Fructose 6-phosphate	0.015 ± 0.002	0.028 ± 0.006	0.025 ± 0.006
Fructose 1,6-bisphosphate	0.028 ± 0.006	0.023 ± 0.003	0.046 ± 0.006
	Activity (nmol/min per g wet wt. of skin)		
	Control	Starved	Re-fed
Synthase I	3.9 ± 0.2	3.6 ± 0.2	3.5 ± 0.2
Synthase D	34.1 ± 1.3	34.0 ± 1.9	33.0 ± 2.2
Phosphorylase a	705 ± 40	702 ± 32	356 ± 22
Phosphorylase b	212 ± 9	238 ± 13	476 ± 12

Effect of starvation and re-feeding on the glucose and glycogen contents of mouse tail skin

The results of these experiments are summarized in Fig. 2, which shows that, after 3 h deprivation of food, skin glycogen was decreased to about 25% of the quantity present in fed mice. Little further change took place when starvation was prolonged to 24 h, even though during this period the mice lost about 15% of their initial body weight. On re-feeding of the mice, skin glycogen content was restored, although the initial rate of resynthesis ($0.37 \mu\text{mol/h}$ per g wet wt. of skin) was significantly slower than the initial rate of depletion ($1.25 \mu\text{mol/h}$ per g wet wt. of skin). Fig. 2 also shows that skin glucose was decreased during starvation and rose on re-feeding, and that the time course of these changes was similar to the changes in blood glucose concentration.

Effects of starvation and re-feeding on metabolic intermediates and on glycogen synthase and phosphorylase activities

Table 1 shows that after 24 h starvation, even though the amount of glycogen in the skin had been decreased by about two-thirds, the concentration of UDP-glucose and the activities of glycogen synthase I and D and of phosphorylase a and b were identical with control values. However, the findings that, in spite of the decrease in glycogen and glucose, the pool of hexose monophosphate increased from 0.078 to $0.145 \mu\text{mol/g}$ wet wt. of skin ($P = 0.001$) and that the concentration of fructose 1,6-bisphosphate was not significantly different from control values suggest that glycolysis was inhibited at the stage of phosphofructokinase, and taken

together these results indicate that, in common with many other tissues, the utilization of carbohydrate in skin is restricted in starvation and that other fuels are preferentially utilized.

In view of the sensitivity of skin glycogen to short periods of food deprivation (Fig. 2) and the variable nature of the feeding habits of individual mice, it was not practicable to measure the concentrations of metabolites and the activities of glycogen synthase and phosphorylase during the phase of glycogen depletion. However, after 24 h starvation mice began to feed simultaneously when presented with food, and in consequence these measurements were made 1.5 h later, when skin glycogen contents were rising rapidly. In spite of the transition to a phase of net glycogen synthesis, there was no change in glycogen synthase I and D activities, but the concentration of UDP-glucose increased from 0.072 to $0.113 \mu\text{mol/g}$ wet wt. ($P = 0.01$), and the proportion of phosphorylase in the a form fell from 75% to 44% ($P = 0.001$). Table 1 also shows that during the transition from the starved to the fed state the hexose monophosphate pool remained high in spite of the enhanced efflux from the pool owing to both net glycogen synthesis and the enhanced rate of glycolysis, which is indicated by the finding that the concentration of fructose 1,6-bisphosphate increased from 0.023 to $0.046 \mu\text{mol/g}$ wet wt. ($P = 0.05$).

Thus it seems that in mouse tail skin, on re-feeding, the restricted glucose utilization characteristic of the starved state is succeeded by a phase of rapid glucose utilization, and, although there is both enzymic and isotopic evidence that glycconeogenesis may occur in rodent skin (Peters & White, 1976),

the rate appears to be low, and it is more probable that the changes described reflect variations in the uptake of circulating glucose (Fig. 2). However this may be, the data support the view that skin glycogen is replenished partly by restricting its degradation (mediated by a decrease in the proportion of phosphorylase in the *a* form) and partly by enhanced synthesis driven by a rise in the concentration of UDP-glucose. These results show that in skin an increase in the proportion of glycogen synthase in the I form is not an obligatory precondition for net glycogen synthesis, but that under the experimental conditions described the rate of synthesis may be determined by the availability of substrate. It is also noteworthy that, in spite of the constancy of the activation state of glycogen synthase, there was a large decrease in the proportion of phosphorylase in the *a* form. However, in considering the significance of these findings it should be emphasized that whole skin is a complex tissue, and that even within the epidermis many histochemical studies have shown that the distribution of glycogen is not uniform, and it seems possible that the same may be true of the enzymes concerned in its metabolism.

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References

- Bergmeyer, H.-U. & Michal, G. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H.-U., ed.), 2nd edn., vol. 3, pp. 1233–1237, Academic Press, New York and London
- Burton, K. (1968) *Methods Enzymol.* **12B**, 163–165
- Campbell, A. K. & Simpson, J. S. (1979) in *Techniques in Life Sciences* (Kornberg, H. L., Metcalfe, J. C., Northcote, D. H., Pogson, C. I. & Tipton, K. F., eds.), vol. B213, pp. 1–56, Elsevier, Amsterdam
- Cohen, P. (1980) *Mol. Aspects Cell. Regul.* **1**, 1–10
- Halprin, K. M. & Ohkawara, A. (1966) *J. Invest. Dermatol.* **46**, 51–69
- Halprin, K. M., Ohkawara, A. & Levine, V. (1973) *Arch. Dermatol.* **107**, 706–711
- Hers, H.-G. (1976) *Annu. Rev. Biochem.* **45**, 167–189
- Im, M. J. C. & Hoopes, J. E. (1970) *J. Surg. Res.* **10**, 459–464
- Kemp, A. & Kits van Heijningen, J. M. (1954) *Biochem. J.* **56**, 646–648
- Keppler, D. & Decker, K. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H.-U., ed.), 2nd edn., vol. 4, pp. 2222–2228, Academic Press, New York and London
- Lang, G. & Michal, G. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H.-U., ed.), 2nd edn., vol. 3, pp. 1238–1242, Academic Press, New York and London
- Lobitz, W. C., Brophy, D., Larner, A. E. & Daniels, F. (1962) *Arch. Dermatol.* **86**, 207–211
- Michal, G. & Beutler, H.-O. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H.-U., ed.), 2nd edn., vol. 3, pp. 1314–1319, Academic Press, New York and London
- Ohkawara, A., Halprin, K. M. & Levine, V. (1972) *J. Invest. Dermatol.* **59**, 264–268
- Oliver, I. T. (1961) *Nature (London)* **190**, 810–811
- Peters, R. F. & White, A. M. (1976) *Biochem. J.* **156**, 465–468
- Peters, R. F. & White, A. M. (1978) *Br. J. Dermatol.* **98**, 301–314
- Stalmans, W. & Hers, H.-G. (1975) *Eur. J. Biochem.* **54**, 341–350
- Thomas, J. A., Schlender, K. K. & Larner, J. (1968) *Anal. Biochem.* **25**, 486–499
- van Handel, E. (1965) *Anal. Biochem.* **11**, 256–265
- Williams, J. P. G. (1972) *Cell Differ.* **1**, 317–323
- Willstätter, R. & Rhodewald, M. (1934) *Hoppe-Seyler's Z. Physiol. Chem.* **225**, 103–124