2. Each amino acid was incorporated into the protein of the membrane, and the glycerol into the lipid, without giving rise to other residues.

3. The presence of high concentrations of penicillin had no effect on the rates of incorporation.

4. Both novobiocin and streptomycin inhibited the incorporation of amino acids, and, to a much smaller extent, of glycerol; vancomycin inhibited the incorporation of amino acids and glycerol equally. There was no evidence for a selective inhibition, by any of these antibiotics, of incorporation into the membrane fraction compared with the total cell protein and lipid.

I am indebted to Dr K. McQuillen and Dr B. D. Davis for their interest and encouragement. I thank the Medical Research Council for a Scholarship, and the Commonwealth Fund, New York, for a Harkness Fellowship. This investigation was supported in part by grant G-9078 from the National Science Foundation to Dr B. D. Davis.

REFERENCES

Anand, N. & Davis, B. D. (1960). *Nature, Lond.*, **185**, 22.
Brock, T. D. & Brock, M. L. (1959). *Arch. Biochem. Biophys.* **85**, 176.
Davis, B. D. & Feingold, D. S. (1962). In *The Bacteria*, vol. 4, p. 343. Ed. by Gunsalus, I. C. & Stanier, R. Y. New York: Academic Press Inc.
Dubin, D. T. & Davis, B. D. (1961). *Biochim. biophys. Acta*, **52**, 400.
Fitz-James, P. C. (1958). *J. biophys. biochem. Cytol.* **4**, 257.
Lederberg, J. (1957). *J. Bact.* **73**, 144.
McQuillen, K. (1955). *Biochim. biophys. Acta*, **17**, 382.
McQuillen, K. (1956). *Symp. Soc. gen. Microbiol.* **6**, 127.
McQuillen, K. & Roberts, R. B. (1954). *J. biol. Chem.* **207**, 81.
Park, J. T. & Strominger, J. L. (1957). *Science*, **125**, 99.
Prestidge, L. S. & Pardee, A. B. (1957). *J. Bact.* **74**, 48.
Reynolds, P. E. (1961). *Biochim. biophys. Acta*, **52**, 403.
Reynolds, P. E. (1962). *Biochem. J.* **84**, 99P.
Roberts, R. B., Abelson, P. H., Cowie, D. B., Bolton, E. T. & Britten, R. J. (1955). *Studies in Biosynthesis in* Escherichia coli, p. 36. Washington: Carnegie Institution of Washington.
Salton, M. R. J. (1960). *Microbial Cell Walls*, p. 64. New York: John Wiley and Sons Inc.
Shockman, G. D. & Lampen, J. O. (1962). *J. Bact.* **84**, 508.
Weibull, C. (1957). *Acta chem. scand.* **11**, 881.
Yudkin, M. D. (1962). *Biochem. J.* **82**, 40P.
Yudkin, M. D. (1963). Ph.D. thesis: University of Cambridge.

Biochem. J. (1963) **89**, 296

'Glucose 6-Phosphate-Dehydrogenase' Activity and Thiol Content of Thymus Nuclei from Control and X-Irradiated Rats

By HELEN DEAKIN, MARGERY G. ORD and L. A. STOCKEN
Department of Biochemistry, University of Oxford

(*Received* 22 *March* 1963)

Creasey & Stocken (1959) showed that the formation of acid-labile phosphate by nuclei isolated from rat thymus gland was completely prevented by exposing the animals to a 100 r. dose of X-rays. The possible function of 'glucose 6-phosphate dehydrogenase' (EC 1.1.1.49) in this nuclear phosphorylation (Ord & Stocken, 1962*a*) led to an examination of the activity of the enzyme after irradiation. The results suggested that alterations in the concentration of thiol (SH) compounds in the nuclei from normal rats might influence the activity of the dehydrogenase and that such alterations might be produced by X-irradiation. Glutathione and glutathione reductase (EC 1.6.4.2) have been described in nuclei from calf thymus (Stern & Timonen, 1954; Wang, 1962), and a study of nuclear thiol groups has been made by Klouwen (1962). The present paper indicates how the method of preparation of the nuclei affects the nature and amount of SH compounds found. The interactions between 'glucose 6-phosphate dehydrogenase' and glutathione reductase have also been examined and shown to complicate the analysis of the effects of X-irradiation on the nuclei.

METHODS

Animals. These were taken from the laboratory strain of Wistar rats. Whenever possible we used females that were put from the time of weaning into a room with a 12 hr. light period, from 8 a.m. to 8 p.m. In all cases the animals were of 100–140 g. body weight (4–6 weeks old).

Nuclei. These were prepared as described by Ord & Stocken (1961) in either 'ionic medium' [KCl (85 mM), NaCl (8·5 mM), $CaCl_2$ (2·5 mM), $MgCl_2$ (2·5 mM) and triethanolamine hydrochloride (5 mM), adjusted to pH 7·2] or in 'sucrose medium' [sucrose (0·25M), $CaCl_2$ (5 mM) and

tris (5 mM), at pH 7·2]. In some experiments the nuclei were prepared in sucrose medium and finally suspended in ionic medium. The final concentration of the suspension was usually the equivalent of one thymus/ml. of medium.

'Glucose 6-phosphate dehydrogenase'. 'Glucose 6-phosphate-dehydrogenase' activity was determined spectrophotometrically, in the presence of 1 mM-glucose 6-phosphate and 0·1 mM-NADP$^+$, from the increase in absorption at 340 mμ produced by NADPH. A portion (2 ml.) of nuclear suspension was added to 2 ml. of medium containing the substrates and centrifuged immediately at 3500g for 1 min. The absorption was measured at 1–2 min. intervals for 10–12 min. at room temperature (18°). The absorption increased linearly for 15–20 min. No attempt was made to separate glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (EC 1.1.1.44).

Glutathione reductase. This was determined by the decrease in absorption at 340 mμ in the presence of 0·1 mM-oxidized glutathione (GSSG) and 0·1 mM-NADPH. The procedure was as described for 'glucose 6-phosphate dehydrogenase'.

Thiols. These were assayed with either *p*-chloromercuribenzoate (Boyer, 1954) at pH 7·2 or with di-(2-carboxy-3-nitrophenyl) disulphide (distributors: R. N. Emmanuel Ltd., London) (Ellman, 1959). Reduced glutathione (GSH) (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.) was used as a standard. A portion (0·5 ml.) of nuclear suspension was diluted with either 2·5 ml. of medium and 0·02 ml. of 10 mM-di-(2-carboxy-3-nitrophenyl) disulphide or 1·5 ml. of medium and 1·0 ml. of 0·3 mM-*p*-chloromercuribenzoate dissolved in medium. After various times of incubation at room temperature (18°) the suspensions were centrifuged at 3500g for 1 min. and the supernatants removed for spectrophotometric readings at 412 or 255 mμ respectively.

Adenosine triphosphate and pyrophosphate. The ATP and pyrophosphate in the nuclear preparations were measured after deproteinization with trichloroacetic acid and removal of the acid with ether. ATP was determined by the method of Holton (1959) and pyrophosphate by the use of pyrophosphatase (Bailey & Webb, 1944).

Deoxyribonucleic acid. DNA was determined by the diphenylamine method of Burton (1956).

Lysine-rich and arginine-rich histones. These were isolated from nuclei according to the method of Daly & Mirsky (1954–55).

Emulsions of linoleic acid peroxide. These were prepared by oxygenation for 20 min. at room temperature (18°) of linoleic acid in sucrose medium (cf. Lewis & Wills, 1962). The peroxide content was determined as described by Wagner, Clever & Peters (1947).

Radioautography. This was performed as described by Fitzgerald, Simmel, Weinstein & Martin (1953). '[1-^{14}C]-*N*-Ethylmaleimide' (0·05 μc; specific activity 8 μc/mg.; from Schwartz Bio-Research Inc., Mount Vernon, N.Y., U.S.A.) was incubated with 0·3 ml. of nuclear suspension and diluted to 1 ml. with medium. After 0, 15 or 60 min. at room temperature the suspensions were centrifuged and the nuclear residues were washed twice with unlabelled *N*-ethylmaleimide (2 ml.; 50 mg./100 ml. of medium). The nuclei were then suspended in 1 ml. of medium, 0·5 ml. was withdrawn and plated on to aluminium planchets for determination of ^{14}C activity (Nuclear–Chicago gas-flow counter), and the remainder was used for radioautography. Kodak AR-10 stripping film was used and 3–4 weeks were allowed for exposure. In experiments with nuclei in sucrose medium the ^{14}C activity in 0·05–0·1 ml. of the final suspension was assayed by scintillation counting (type 6012; Isotope Developments Ltd.).

X-irradiation. Exposures of 200 r. or less *in vivo* were given in the Department of Radiotherapy, the Churchill Hospital, Oxford. The characteristics were: half-value layer, 1·1 mm. Cu; peak voltage, 220 kv at 15 mA. In most of the experiments with 1000 r. *in vivo* the irradiations were performed at the Medical Research Council Radiobiological Research Unit, Harwell. Here the characteristics were: half-value layer, 2·5 mm. Cu; peak voltage, 250 kv at 14 mA. The irradiations *in vitro* were carried out in the University Department of Botany. Characteristics were: unfiltered, 70 kv at 5 mA.

RESULTS

'Glucose 6-phosphate dehydrogenase' in thymus nuclei. The confirmation of the radiosensitivity of nuclear phosphorylation (Ord & Stocken, 1962*b*) prompted an examination of enzyme systems that might be involved in the phosphorylation and that might be affected by X-irradiation. 'Glucose 6-phosphate-dehydrogenase' activity appeared to be diminished in nuclei isolated in ionic medium from thymus glands of rats that had received 88–100 r. 15 min. earlier (Table 1). A dose of 5000 r. *in vitro* did not inhibit the dehydrogenase activity in a rat-liver homogenate (Kunkel, Höhne, Maas & Schubert, 1955), so that direct inactivation of the enzyme itself is unlikely. The nuclear enzyme was not affected by the presence of pancreatic or micrococcal deoxyribonucleases nor by pancreatic ribonuclease (M. G. Ord & L. A. Stocken, unpublished work), so that damage to the enzyme through the release of nucleases (Bacq & Alexander, 1961) was improbable. The range of activity found in both control (Table 1) and irradiated nuclear preparations was, however, disturbingly broad, and a critical examination was therefore undertaken of factors that might alter the activity.

It was observed that, after nuclei had been prepared in ionic medium, the activity of the enzyme was increased by nearly 100% (Table 1) by storing the preparation at 0° for 30 min. before centrifuging. In the experiments with the dehydrogenase obtained from irradiated rats, care was taken that the nuclear suspension was assayed immediately after isolation of nuclei. Centrifuging of a freshly prepared nuclear suspension in a Spinco rotor SW 39 (38000 rev./min. for 15 min.) did not increase the activity in the supernatant, but, when the nuclear sediment obtained after centrifuging for 1 min. at 3500g was resuspended and the activity in the resulting (second) supernatant measured, 'glucose 6-phosphate dehydrogenase' was again detectable with an activity of about half that

present in the first supernatant. These results showed that the dehydrogenase was fairly readily extracted from nuclei prepared in ionic medium. The decrease in enzyme activity found after resuspension of the nuclei may explain the decreased phosphorylation found in twice-washed nuclei prepared in ionic medium (Ord & Stocken, 1962*a*).

Since these results suggested that the dehydrogenase activity might be due to absorption of cytoplasmic components, nuclei were prepared in sucrose medium. J. A. Smit & L. A. Stocken (unpublished work) have found a greater loss of protein from nuclei prepared in ionic medium than from nuclei prepared in sucrose medium. Higher activity of 'glucose 6-phosphate dehydrogenase' in sucrose medium (Table 1) supports their finding and, since there was no increase in extractability of the enzyme after standing, it seems that the enzyme is more firmly bound than in nuclei prepared in ionic medium. This was confirmed when nuclei that had been prepared in sucrose medium were subsequently suspended in ionic medium and allowed to stand for 10 min. at room temperature before centrifuging. This gave a greater dehydrogenase activity in the extract than by the other methods. Though these results indicated that great care was required in the preparation of nuclei to obtain reproducible results, further experiments showed that interference in the measurement of the enzyme could arise because of the presence in the supernatants of enzyme systems that used NADPH.

Table 1. '*Glucose 6-phosphate-dehydrogenase*' *activity in nuclei obtained from rat thymus gland*

Five animals were used/nuclear preparation. A portion (2 ml.) of the nuclear suspension was diluted with 2 ml. of incubation mixture and the production of NADPH was followed spectrophotometrically for 10–15 min. The numbers of experiments are given in parentheses. Activity is expressed as μm-moles of NADPH formed/min./mg. of DNA phosphorus at 18°.

	Activity
Nuclei prepared in ionic medium:	
Control (12)	$1{\cdot}35 \pm 0{\cdot}485$
Nuclei from rats which received 88–100 r. 15 min. earlier (6)	$1{\cdot}09 \pm 0{\cdot}420$
Nuclei sedimented at 3500*g*-min. (1)	1·51
Nuclei sedimented at 10^6*g*-min. (1)	1·51
Nuclei washed once (1)	1·72
Nuclei washed twice (1)	0·88
After standing for 30 min. at 0° (2)	2·58
Nuclei prepared in sucrose medium:	
Control (5)	$2{\cdot}98 \pm 0{\cdot}611$
Nuclei extracted at 0° (2)	0·66
After standing 30 min. at 0° (2)	2·98
Nuclei extracted at 18° with ionic medium (2)	10·46

GSSG (0·1–1·0 mM) lowered the apparent activity of the dehydrogenase (Table 2) but this effect was considerably diminished by 10 μM-Zn^{2+} ion, which inhibits glutathione reductase (Fawaz & Fawaz, 1962). The presence of this enzyme in the nuclear extracts was confirmed by direct assay with 0·1 mM-GSSG and NADPH.

Thiol content of nuclei. The adverse effects of GSSG on the measurement of dehydrogenase activity suggested that the effects of X-irradiation might be caused by radiochemical oxidation of GSH present in the nuclei.

Determination of the total thiol content of nuclei prepared in ionic medium at pH 7·2 showed that deproteinized extracts contained only small and irreproducible proportions of the total thiol content. The methods of Boyer (1954) and Ellman (1959) gave the same values for total thiol content on identical samples of nuclei prepared in ionic medium. The reaction was comparatively slow, especially with *p*-chloromercuribenzoate, when the reaction was not complete until 30–45 min. after the addition of the reagent. The addition of 10 μM-Zn^{2+} ion had no effect on the concentration of thiol

Table 2. '*Glucose 6-phosphate dehydrogenase*' *and glutathione reductase interactions in nuclei from rat thymus gland*

In the first group of experiments nuclei were prepared in ionic medium, and in the second group they were isolated in sucrose medium and extracted with ionic medium. Five animals were used for each preparation. Enzyme activity is expressed as μmoles of NADPH produced or utilized/min./mg. of DNA phosphorus at 18°.

Substrate and coenzyme	Variant	Enzyme activity (control)	Effect of variant (% of control)
Group 1			
1 mM-Glucose 6-phosphate + 0·1 mM-$NADP^+$	+ 1 mM-GSSG	1·35	30 (4)
	+ 0·1 mM-GSSG	1·35	68 (4)
	+ 10 μM-GSSG	1·35	100 (1)
Group 2			
1 mM-Glucose 6-phosphate + 0·1 mM-$NADP^+$	+ 10 μM-$ZnSO_4$	10·13	126
	+ 0·1 mM-GSSG	10·13	74
	+ 0·1 mM-GSSG + 10 μM-$ZnSO_4$	10·13	110
1 mM-Glucose 6-phosphate + 0·1 mM-$NADP^+$	+ 10 μM-$ZnSO_4$	10·8	117
0·1 mM-GSSG + 0·1 mM-NADPH	+ 10 μM-$ZnSO_4$	1·34	0
	+ 1 mM-Glucose 6-phosphate	1·34	0

groups, and chromatographic analysis of acid extracts of the nuclei failed to establish the presence of reproducible amounts of glutathione.

Isolation of lysine-rich and arginine-rich histones showed that the SH content of nuclei prepared in ionic medium was mainly attributable to the arginine-rich histone, and, in agreement with previous work (Daly & Mirsky, 1954–55), no thiol groups were detectable in the lysine-rich fraction. Of the total SH content 67 % was associated with the arginine-rich histone fraction and 83 % of the SH groups of the histone reacted with di-(2-carboxy-3-nitrophenyl) disulphide in 5 min. The low recovery of SH may be due to the presence of slowly reacting thiol compounds that are not extracted from the nuclei by acid treatment.

Nuclei prepared in sucrose medium contained very much higher amounts of SH groups (Table 3), as might be expected from the greater loss of proteins from nuclei isolated in ionic medium. Dialysis of the supernatant obtained when nuclei prepared in sucrose were extracted with ionic medium showed that diffusible thiols were present. Chromatographic analysis of the acid extract from nuclei prepared in sucrose medium indicated the presence of glutathione as well as higher-molecular-weight SH compounds. It therefore seems probable that preparations of nuclei in ionic medium did not allow the retention of reproducible amounts of associated glutathione.

The effects of 10 μM-Zn^{2+} ion on the total SH content of nuclei isolated in sucrose medium (Table 3) and on the 'glucose 6-phosphate-dehydrogenase' activity (Table 2) suggest that these nuclei also contained small amounts of GSSG, whose reduction would normally be catalysed in the extracts by glutathione reductase and NADPH. Nuclei prepared in ionic medium contain all four species of nicotinamide coenzyme (M. G. Ord & L. A. Stocken, unpublished work). The presence of Zn^{2+} ions did not abolish the slowly reacting component in the thiol reaction. Of the SH content of nuclei prepared in sucrose medium 69 % was associated with the arginine-rich histone fraction.

Table 3. *Thiol content of nuclei isolated from rat thymus gland*

Nuclei were incubated at pH 7·2 with di-(2-carboxy-3-nitrophenyl) disulphide or *p*-chloromercuribenzoate at 18°. After 5–45 min. the preparations were centrifuged at 3500*g* for 1 min. and the supernatants used for spectrophotometric assay. The numbers of experiments are given in parentheses. Thiol content is expressed as μm-moles of SH/mg. of DNA phosphorus.

	Thiol content	
	Ellman (1959) method	Boyer (1954) method
Nuclei prepared in ionic medium:		
Total SH	308 ± 38 (15)	280 (5)
SH reacting in 5 min.	255 (7)	84 (2)
Nuclei prepared in sucrose medium:		
Total SH	469 ± 33 (7)	—
SH reacting in 5 min.	375 (5)	—
Effect of $ZnSO_4$		
Control	525, 426	—
+ 10 μM-$ZnSO_4$	482, 399	—
Nuclei prepared in sucrose and suspended in ionic medium:		
Total SH	666, 620	—

In view of the effects of X-irradiation *in vivo* on nuclei isolated in ionic medium, it was interesting that the highest thiol contents were found with nuclei that had been prepared in sucrose and extracted with ionic medium.

Effect of X-irradiation on the thiol content of nuclei. The potential interactions between glutathione reductase and disulphide compounds present or produced in nuclei made it advantageous to investigate the effects of X-irradiation *in vitro* when controls could be directly compared and when glutathione reductase could be inhibited by Zn^{2+} ions. Irradiation with 200 r. *in vitro* had no detectable effect on the thiol content of 10 μM-GSH in 0·02 M-phosphate buffer, pH 7·2, but 1000 r. decreased the concentration by 43 % (cf. Barron & Flood, 1950). Irradiation of nuclei prepared in ionic medium failed to show any effect on their total SH content, but if nuclei were prepared in sucrose a decrease in SH content was apparent (Table 4). If no Zn^{2+} ions were present the decrease in thiol content was only found at the first spectrophotometric reading (total time from the end of irradiation to measurement, 10 min.), but when 10 μM-Zn^{2+} ion was present the decrease was apparent at both time-intervals and was indeed still found in nuclei that had been kept at 0° for 30–45 min. after irradiation. This suggested that oxidized thiol compounds could be reconverted into the reduced form by the glutathione reductase present in the preparations.

An attempt was made to isolate the thiol compound that might have been oxidized. A dose of 1000 r. was given to 5 ml. of nuclei prepared in sucrose medium. The control and irradiated preparations were treated with 1 ml. of 30 % (w/v) trichloroacetic acid, and the acid was removed from the deproteinized supernatants with ether. Only 30 % of the total SH content of the nuclei was extracted into the supernatant, and no difference was found between extracts from control and irradiated nuclei. In control preparations of nuclei in sucrose medium about 20 % of their total SH groups remained in the supernatant after centrifuging the suspension for 1 min. at 3500*g*, and this fraction also was unaffected when the nuclei were given 1000 r. Thus when nuclei are suspended in

sucrose medium the radiosensitive thiol components are not readily extractable from the nucleus.

When X-irradiation was given *in vivo* 200 r. produced no significant effect on the thiol content of nuclei isolated in ionic medium, but with 1000 r. increased SH reactivity was found (Table 5). This was confirmed by using ^{14}C-labelled *N*-ethylmaleimide, when the total ^{14}C activity of the washed nuclei increased with the time of incubation with *N*-ethylmaleimide and showed higher counts with nuclei from the irradiated rats. When nuclei were prepared in sucrose medium after X-irradiation *in vivo* two effects were detected. In the absence of Zn^{2+} ions an initial depression in thiol content, found when nuclei were incubated for 5 min. with the reagent, was followed by a slight increase in activity after 30 min. of incubation. This increase was confirmed radioautographically (Table 7), and, when uptake of ^{14}C-labelled *N*-ethylmaleimide was measured, 15 min. and 60 min. of incubation showed 11 and 12 % increases respectively in uptake in the irradiated nuclei. It is assumed that during the lengthier manipulations of the nuclei the glutathione reductase reversed the initial lowering of the SH content. When 10 μM-Zn^{2+} ion was present in the sucrose medium throughout the preparation of the nuclei 1000 r. *in vivo* gave a fall in nuclear SH content which was detectable after both 5 min. and 30 min. of incubation with di-(2-carboxy-3-nitrophenyl) disulphide. In this experiment, as with irradiation *in vitro*, there was no decrease in SH content of that fraction (20 %) of the thiol activity which was present in the supernatant obtained after centrifuging the nuclear preparation for 1 min. at 3500 **g**.

Radioautography of thiol groups. ^{14}C-labelled *N*-ethylmaleimide was incorporated into an insoluble compound clearly localized in the nucleus.

Table 4. *Effect of X-irradiation* in vitro *on the thiol content of nuclei from rat thymus gland*

Nuclei were prepared in sucrose medium. A portion (5 ml.) of the preparation was irradiated at 0° and the thiol content of the control and irradiated suspensions was measured by using di-(2-carboxy-3-nitrophenyl) disulphide. When present, the final concentration of $ZnSO_4$ was 10 μM. Thiol content is expressed as μm-moles of SH/mg. of DNA phosphorus.

Exposure (r.)	$ZnSO_4$	Time of incubation with SH reagent (min.)	Thiol content	
			Control	Irradiated
1000	−	5	431	382
	−	30	486	481
1000	+	5	392	344
	+	30	531	450
1000	+	5	428	360
	+	30	488	412
1000	−	30	492	497
	+		541	511

Table 5. *Effect of X-irradiation* in vivo *on the thiol content of nuclei isolated from rat thymus gland*

The animals were killed 15–60 min. after irradiation, and nuclei were prepared from control and exposed rats. Six rats were used/group. In some of the experiments in which 200 r. was given the SH groups were measured by using both di-(2-carboxy-3-nitrophenyl) disulphide and *p*-chloromercuribenzoate; in the other experiments only the method of Ellman (1959) was used. The numbers of experiments are given in parentheses. Thiol content is expressed as μm-moles of SH/mg. of DNA phosphorus.

Exposure (r.)	Time after end of exposure (min.)	Time of incubation with SH reagent (min.)	Thiol content	
			Control	Irradiated
Nuclei isolated in ionic medium:				
200 (4)	15	25	314	325
1000 (1)	15	25	299	353
1000 (1)	60	25	270	348
Nuclei isolated in sucrose medium:				
1000 (1)	60	5	289	255
1000 (1)	60	30	357	366
1000 (1)	60	5	363	302
(10 μM-$ZnSO_4$ present)		30	551	411
200 (3)	60	30	446	465
(10 μM-$ZnSO_4$ present)				

The number of nuclei showing the presence of label increased with time (Table 6), and from a differential analysis it appeared that the larger thymocytes took up the label more rapidly and to a greater extent than in the small thymocytes (Table 7). The increase in SH content of nuclei isolated in ionic medium after exposing the rats to 1000 r. was due mainly to an increase in SH content of individual nuclei rather than to an increase in the number of nuclei reacting. The action was principally on the small thymocytes, in which the numbers of grains/nucleus appeared to be higher in the irradiated population.

Radiomimetic effects of linoleic acid peroxide and oxidized glutathione. The possibility that lipid peroxides are produced in tissues by X-irradiation has been considered by Horgan, Philpot, Porter & Roodyn (1957), who found evidence for peroxide formation in mice after exposure. Lewis & Wills (1962) have shown that linoleic acid peroxide oxidizes SH groups of cysteine very readily, and GSH and papain more slowly.

Linoleic acid peroxide in the concentration used did not affect the absorption at 412 mμ that had been produced by reaction of di-(2-carboxy-3-nitrophenyl) disulphide and 50 μm-moles of glutathione, but when the peroxide was added immediately before the reagent a slight inhibition was found (Table 8), as described by Lewis & Wills (1962). The SH content of the nuclear preparation was lowered by the addition of linoleic acid peroxide; the nuclear SH components were more sensitive to peroxide than was GSH.

Since oxidation of nuclear thiol groups could be produced by linoleic acid peroxide we decided to see if oxidized SH compounds would have any effect on nuclear phosphorylation. GSSG was used as a model and the ability of the nuclear preparations to phosphorylate was examined by measuring the pyrophosphate formation of the nuclei during

Table 6. *Radioautographic analysis of the uptake of* ^{14}C-*labelled* N-*ethylmaleimide into nuclei isolated in ionic medium from thymus gland of normal and X-irradiated rats*

In Expt. 1, the nuclei were isolated 15 min. after irradiation, and in Expt. 2 60 min. after irradiation. The radiation dose in each experiment was 1000 r.

Expt. no.	Time of incubation with *N*-ethylmaleimide (min.)	Control: No. of nuclei counted	Control: Percentage with 2 or more grains	Irradiated: No. of nuclei counted	Irradiated: Percentage with 2 or more grains
1	0	719	27·8	529	26·0
	15	481	48·0	421	54·8
	60	256	70·7	372	80·4
2	0	568	28·7	583	37·6
	15	639	39·2	626	41·6
	60	583	56·5	487	53·4

Table 7. *Analysis of grain counts in nuclei from thymus gland of control and X-irradiated rats killed* 15 *or* 60 *min. after exposure to* 1000 *r.*

Nuclei were distinguished as small nuclei and 'others'. The small nuclei comprised about 80% of the total population. Nuclei were isolated in ionic medium, except in Expt. 3, when they were isolated in sucrose medium. Unless otherwise stated, the nuclei were incubated for 60 min. with ^{14}C-labelled *N*-ethylmaleimide. In Expt. 1 the rats were killed 15 min. after exposure; in Expts. 2 and 3 they were killed 60 min. after X-irradiation.

Expt.	Type of nuclei	No. of nuclei counted (and those with more than 2 grains)	Percentage of population with grain counts: 2–4	5–7	8–10	> 10
1	'Others'*	41 (32)	56	25	10	9
	'Others'	37 (32)	19	34	19	28
	Small	152 (91)	72	22	6	0
	Undifferentiated controls	(133)	66	21	8	5
	Undifferentiated 1000 r.	(112)	55	36	4	5
2	Small (controls)	(227)	70	23	6	1
	Small (1000 r.)	(241)	65	31	3	1
3	Small (controls)	(143)	53	31	11	5
	Small (1000 r.)	(168)	40	33	20	7

* Incubated with ^{14}C-labelled *N*-ethylmaleimide for less than 1 min.

incubation at 0°, in the presence and absence of GSSG. Pyrophosphate formation exceeding the disappearance of ATP originally present in the nuclear suspensions has been used as a measure of nuclear phosphorylation. The excess of pyrophosphate arises from the utilization of ATP for activation of amino acids, and possibly of other substrates, for synthetic reactions in the nuclei (Ord & Stocken, 1962*a*). In the presence of 1 mM-GSSG the formation of pyrophosphate was diminished to about 30% of that in the control nuclei (Table 9).

DISCUSSION

Ord & Stocken (1961, 1962*a*) showed that nuclei prepared in ionic medium took part in both phosphate transfer reactions and in the activation of amino acids. From the results reported above it is clear that marked loss of protein from the nucleus occurs in this type of preparation (see also Allfrey, 1959) and this presumably accounts for the variability in glucose 6-phosphate-dehydrogenase concentrations in these nuclei. A more important factor in the dehydrogenase assay was interference from the glutathione-reductase system. This system had been investigated in calf-thymus nuclei by Stern & Timonen (1954) but no measurements were reported of total thiol content of the nuclei. Complete analysis of the thiol groups in these preparations has not yet been achieved but the major contribution that arginine-rich histone(s) make to the total thiol content of nuclei from rat thymus was unexpected. Glutathione appears to account for a comparatively small proportion of the total.

The concentration of glutathione reductase probably accounted for the effects of GSSG on 'glucose 6-phosphate dehydrogenase', but the effects of GSSG on nuclear phosphorylation are less easily interpreted. Nuclear phosphorylation requires the presence of oxygen (Allfrey, Mirsky & Osawa, 1955; W. A. Creasey, unpublished work) so that it seems unlikely that NADPH produced by 'glucose 6-phosphate dehydrogenase' is normally completely reoxidized through the glutathione-reductase system. The inhibitory action of GSSG therefore seems attributable either to diversion of NADPH from oxidation which is usually linked to phosphorylation, or to a disturbance produced by GSSG in the normal thiol–disulphide equilibria in nuclear proteins, whose functions are still unknown. Alterations in thiol–disulphide equilibria of nuclear proteins are thought to occur during cell division (see Mazia, 1961); it is nuclei from the larger thymocytes, showing most of the mitotic activity of the thymus (Sainte-Marie & Leblond, 1958), that have the higher SH reactivity in radioautographs.

Two effects of X-irradiation on nuclear thiol groups have been detected. In nuclei isolated in sucrose medium a decrease in SH content was found after irradiation both *in vivo* and *in vitro*. The fall was transient if glutathione reductase was not inhibited. Glutathione reductase is believed to be specific for GSSG (Knox, 1960), but, since GSH was probably a comparatively minor contributor to nuclear SH groups and since interactions between GSH and protein disulphide groups have been described (see Knox, 1960), initial oxidation of SH groups on proteins cannot be ruled out. The second effect of X-irradiation, most obvious in nuclei prepared in ionic medium, was an increase in thiol groups in nuclei from the smaller thymo-

Table 8. *Effect of linoleic acid peroxide on the thiol content of thymus nuclei prepared in sucrose medium*

0·1 M-Linoleic acid in ethanol was diluted with 9 vol. of sucrose medium. After oxygenation of the emulsion for 20 min. at 18°, it was filtered; 0·3 ml. was diluted with 2·2 ml. of medium, and 0·5 ml. of nuclear suspension was added. Approx. 3 min. later 0·02 ml. of di-(2-carboxy-3-nitrophenyl) disulphide was added. The control assays were performed in the presence of 0·3 ml. of oxygenated sucrose containing 10% (v/v) of ethanol. Thiol content is expressed as μm-moles/3 ml.

Peroxide added (μm-moles/3 ml.)	Thiol content (control)	Decrease in thiol content caused by peroxide
Not known	23·5	6·2
20·4	31·1	8·55
27·5	24·6	10·8
27·5	10·6*	1·4

* In this experiment reduced glutathione was used in place of the nuclear preparation.

Table 9. *Effect of oxidized glutathione on pyrophosphate formation by nuclei isolated in ionic medium*

The reaction was stopped by the addition of trichloroacetic acid. ATP and pyrophosphate were determined after the removal of the acid from the deproteinized extracts. The reaction was stopped at 0 min. or after 30 min. of gentle shaking at 0°. Concentrations are expressed as μm-moles/mg. of DNA phosphorus. P_i, Inorganic phosphate; PP, pyrophosphate.

Initial concn. of phosphate		Change in concn. of phosphate after 30 min.					
		Control			+1 mM-GSSG		
ATP	PP	P_i	ATP	PP	P_i	ATP	PP
5·7	17·9	+35·8	−3·4	+23·1	+58·9	−3·9	+5·3
3·5	29·3	+39·2	−0·8	+21·2	+42·5	−0·8	+8·2

cytes. This disturbance probably occurred in both types of preparation, although in nuclei retaining larger amounts of SH compounds the effect was masked by the decrease ascribed to oxidation of histone SH groups. It is not clear whether the two actions of X-rays are related; the increase in SH content was seen in nuclei prepared in ionic medium both at 15 and at 60 min. after irradiation *in vivo*, indicating that it was a fairly immediate effect. It was not, however, detected after irradiation *in vitro* in either type of nuclear preparation. The increased SH content might originate from non-histone thiol groups, which may account for 30% of the total, but SH reactivity can be increased in control nuclei prepared in sucrose if the final suspension is made in ionic medium. This suggests that the higher ionic strength of ionic medium (I 0·11) compared with that of the sucrose medium (I 0·015) might cause partial dissociation of the nucleoprotein structure and permit SH groups that were unavailable in the sucrose medium to become reactive. A similar explanation can be offered for the increased SH content after 1000 r. *in vivo*.

These changes in thiol compounds indicate rather profound alterations in nuclear structure after X-irradiation. Production of SH groups may be the direct result of the ionizing events, but the oxidation may be partly consequential on the formation of lipid peroxides after irradiation (Horgan *et al.* 1957). A decrease in the total SH content of rat thymus after exposure has been reported by Ashwood-Smith (1961), and Kedrova, Antokol'skaya & Rodionov (1961) have found a fall in extractable SH content of liver nuclei after 1500 r. *in vivo*. It is possible that oxidation of histone SH groups may be associated with the protection apparently exerted by the protein of deoxyribonucleoprotein against damage to DNA after X-irradiation *in vivo* and *in vitro* (Peacocke & Preston, 1961). It has already been postulated that the effects of GSSG on nuclear phosphorylation may be through changes in protein thiol–disulphide equilibria, and such changes could also account for the radiosensitivity of this process and might be expected to have an effect on the actual mechanism of spindle formation in mitosis and so provide a biochemical factor in the arrest of this process after X-irradiation.

SUMMARY

1. A study has been made of the thiol groups present in nuclei from rat thymus gland. Of the total thiol content of the nuclei 70% was associated with arginine-rich histones.

2. Proteins, including those containing thiol groups, are lost from nuclei isolated in ionic medium. Nuclei isolated in sucrose medium [sucrose (0·25M), calcium chloride (5 mM), and tris (5 mM), at pH 7·2] retain reproducible amounts of thiol compounds and 'glucose 6-phosphate dehydrogenase'.

3. The reactivity of thiol groups in nuclei isolated in sucrose medium is increased if the final suspension is made in ionic medium.

4. '[1-^{14}C]-N-Ethylmaleimide' has been used in conjunction with radioautography to investigate the distribution of thiol groups in the nuclear population from rat thymus.

5. X-irradiation *in vivo* or *in vitro* decreases the concentration of thiol groups in nuclei isolated in sucrose medium; this effect is transient if the glutathione reductase, present in the nuclei, is not inhibited by 10 μM-Zn^{2+} ion.

6. X-irradiation *in vivo* increases the number of reactive thiol groups especially in nuclei from small thymocytes; this effect is most obvious in nuclei isolated in ionic medium where the inhibitory action of X-irradiation on nuclear thiol groups is not detected.

7. Linoleic acid peroxide, in concentrations approximately equimolar to the thiol concentration, decreased the thiol content of the nuclei.

We acknowledge financial assistance from the Department of Industrial and Scientific Research, the Rockefeller Foundation and the National Institutes of Health, United States Public Health Service (grant no. A-3369. C3). We are grateful to those who performed the irradiations and to Dr W. Bartley for informative discussions. Mr A. Morris and Mr P. Copper gave us unflagging assistance.

REFERENCES

Allfrey, V. G. (1959). In *The Cell*, vol. 1, p. 193. Ed. by Brachet, J. & Mirsky, A. E. New York: Academic Press Inc.

Allfrey, V. G., Mirsky, A. E. & Osawa, S. (1955). *Nature, Lond.*, **176**, 1042.

Ashwood-Smith, M. J. (1961). *Int. J. Radiat. Biol.* **3**, 125.

Bacq, Z. M. & Alexander, P. (1961). *Fundamentals of Radiobiology*, 2nd ed., p. 272. Oxford: Pergamon Press Ltd.

Bailey, K. & Webb, E. C. (1944). *Biochem. J.* **38**, 394.

Barron, E. S. G. & Flood, V. (1950). *J. gen. Physiol.* **33**, 229.

Boyer, P. D. (1954). *J. Amer. chem. Soc.* **76**, 4331.

Burton, K. (1956). *Biochem. J.* **62**, 315.

Creasey, W. A. & Stocken, L. A. (1959). *Biochem. J.* **72**, 519.

Daly, M. M. & Mirsky, A. E. (1954–55). *Amer. J. Physiol.* **38**, 405.

Ellman, G. L. (1959). *Arch. Biochem. Biophys.* **82**, 70.

Fawaz, E. N. & Fawaz, G. (1962). *Biochem. J.* **83**, 438.

Fitzgerald, P. J., Simmel, E., Weinstein, J. & Martin, C. (1953). *Lab. Invest.* **2**, 181.

Holton, P. W. (1959). *J. Physiol.* **145**, 494.

Horgan, V. J., Philpot, J. St L., Porter, B. W. & Roodyn, D. B. (1957). *Biochem. J.* **67**, 551.

Kedrova, B. M., Antokol'skaya, Zh. A. & Rodionov, V. M. (1961). *Biokhimia*, **26**, 234.

Klouwen, H. M. (1962). *Arch. Biochem. Biophys.* **99**, 116.
Knox, W. E. (1960). In *The Enzymes*, 2nd ed., vol. 2, p. 253. Ed. by Boyer, P. D., Lardy, H. & Myrbäck, K. New York: Academic Press Inc.
Kunkel, H. A., Höhne, G., Maas, H. & Schubert, G. (1955). *Progress in Radiobiology*, p. 52.
Lewis, S. E. & Wills, E. D. (1962). *Biochem. Pharmacol.* **11**, 901.
Mazia, D. (1961). In *The Cell*, vol. 3, p. 77. Ed. by Brachet, J. & Mirsky, A. E. New York: Academic Press Inc.
Ord, M. G. & Stocken, L. A. (1961). *Biochem. J.* **81**, 1.
Ord, M. G. & Stocken, L. A. (1962*a*). *Biochem. J.* **84**, 593.
Ord, M. G. & Stocken, L. A. (1962*b*). *Biochem. J.* **84**, 600.
Peacocke, A. R. & Preston, B. N. (1961). *Nature, Lond.*, **192**, 228.
Sainte-Marie, G. & Leblond, C. P. (1958). *Proc. Soc. exp. Biol., N.Y.*, **97**, 263.
Stern, H. & Timonen, S. (1954). *J. gen. Physiol.* **38**, 41.
Wagner, C. D., Clever, H. L. & Peters, F. D. (1947). *Analyt. Chem.* **19**, 980.
Wang, T. Y. (1962). *Nature, Lond.*, **195**, 1099.

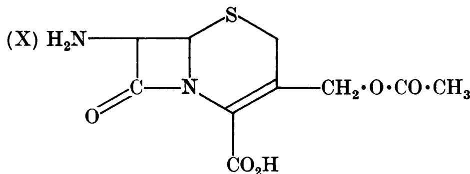
Biochem. J. (1963) **89**, 304

The Formation of Metabolites from Cephalosporin Compounds

BY CYNTHIA H. O'CALLAGHAN AND P. W. MUGGLETON
Glaxo Research Ltd., Stoke Poges, Buckinghamshire, and Greenford, Middlesex

(*Received* 14 *March* 1963)

The discovery of cephalosporin and the elucidation of its structure has led to the preparation of a new series of compounds with antibacterial activity. The nucleus of the cephalosporins consists of a dihydrothiazine ring fused to a β-lactam ring (Abraham & Newton, 1961).



Cephalosporin C has an α-aminoadipoyl group in the 7-position (X) and is the only member of this series of compounds that has so far been found in natural products. The cephalosporins differ from the penicillins in that no analogues are produced when precursors such as phenylacetic acid are incorporated in the fermentation medium. The α-aminoadipoyl group can, however, be removed from cephalosporin C by hydrolysis (Loder, Newton & Abraham, 1961) or by treatment with nitrosyl chloride (Morin, Jackson, Flynn & Roeske, 1962), and the 7-amino group can then be substituted if desired. The first cephalosporin analogue to be prepared in this way was 7-phenylacetamidocephalosporanic acid (sodium salt) (called 'phenylacetyl cephalosporin').

In laboratory studies, which are not reported in detail below, we have shown that phenylacetyl cephalosporin has an antibacterial spectrum similar to that of benzylpenicillin, with the added advantages of a greatly increased stability to acid and to penicillinase. Against experimental *Staphylococcus aureus* infections in mice, phenylacetyl cephalosporin gave irregular results in which the protection obtained was not parallel with the dose given. Although it has a low toxicity for mice, some animals receiving low doses were protected, whereas some having larger doses died.

To explain these irregular results, in which no clear-cut end points were obtained, a variable metabolic decomposition of the substance to a compound with decreased antibacterial activity was postulated, and the work reported below describes the investigation of the fate of phenylacetyl cephalosporin and other cephalosporins in the mammalian body.

EXPERIMENTAL

Chromatography and bio-autographs. A suitable solution in 30 μl. quantities was applied to Whatman no. 1 papers, buffered with 0·05M-sodium phosphate at pH 6·0. Undiluted serum and urine samples were applied to the paper in the same volume but, where it was expected that the antibiotic concentration would be low, several (up to five) applications were made on the same spot, the paper being dried in a stream of warm air between applications. The solvents used were: (*a*) butan-1-ol–ethanol–water (4:1:5, by vol.); (*b*) propan-1-ol–water (7:3, v/v). The chromatograms (descending) were allowed to run for 18 hr. After drying, the papers were placed on nutrient–agar plates seeded, for phenylacetyl cephalosporin, with 1% of a 24 hr. broth culture, diluted 1:100, of *S. aureus* (NCTC 7447). For cephalosporin C or *N*-dinitrophenyl cephalosporin C, 1% of a 1:500 dilution of a spore suspension of *Bacillus subtilis* (ATCC 6633) was used. The papers were removed after 15 min., and the plates were incubated at