- Crampton, C. F., Reller, H. H. & Haurowitz, F. (1953). *J. Immunol.* **71**, 319.
- Dixon, F. J., Bukantz, S. C. & Dammin, G. J. (1951). *Science*, **113**, 274.
- Fraenkel-Conrat, H. & Williams, R. C. (1955). *Proc. nat. Acad. Sci., Wash.*, **41**, 690.
- Francis, G. E. & Hawkins, J. D. (1957). *Biochem. J.* **65**, 570.
- Francis, G. E., Hawkins, J. D. & Wormall, A. (1957). *Biochem. J.* **65**, 560.
- Garvey, J. S. & Campbell, D. H. (1954). *J. Immunol.* **72**, 131.
- Garvey, J. S. & Campbell, D. H. (1957). *J. exp. Med.* **105**, 361.
- Haurowitz, F. (1953). *Annu. Rev. Microbiol.* **7**, 389.
- Haurowitz, F. (1960). *Annu. Rev. Biochem.* **29**, 609.
- Haurowitz, F. & Crampton, C. F. (1952). *J. Immunol.* **68**, 73.
- Haurowitz, F. & Walter, H. (1955). *Proc. Soc. exp. Biol., N.Y.*, **88**, 67.
- Hawkins, J. D. & Haurowitz, F. (1959). *Biochem. J.* **72**, 5 p.
- Ingraham, J. S. (1951). *J. infect. Dis.* **89**, 109.
- Kay, E. R. M. & Dounce, A. C. (1953). *J. Amer. chem. Soc.* **75**, 4041.
- Levine, S. (1954). *Arch. Biochem. Biophys.* **50**, 515.
- McMaster, P. D. & Kruse, H. (1951). *J. exp. Med.* **94**, 323.
- Proom, H. (1943). *J. Path. Bact.* **55**, 419.
- Spector, S. (1956). *Handbook of Biological Data*, p. 340. Philadelphia: W. B. Saunders Co.
- Steinberg, D. (1959). *Nature, Lond.*, **183**, 1253.
- Talmage, D. W., Dixon, F. J., Bukantz, S. C. & Dammin, G. J. (1951). *J. Immunol.* **67**, 243.
- Weigle, W. O. & Dixon, F. J. (1957). *J. Immunol.* **79**, 24.

Biochem. J. (1961) **80**, 210

Possible Sites of Catabolism of Rat Serum Albumin

By J. D. HAWKINS

Department of Biochemistry and Chemistry, Medical College of St Bartholomew's Hospital, London, E.C. 1

(Received 27 September 1960)

Many studies have been made of the turnover of mammalian serum proteins, generally with isotopically labelled proteins, but very little information is available about the sites of breakdown. Cohen & Gordon (1958) have demonstrated the catabolism of rat serum albumin by the perfused liver and have concluded that this organ can catabolize only about 13–15% of the protein which is broken down in the intact animal.

In the course of experiments reported in the preceding paper (Hawkins & Haurowitz, 1961), rats were killed at various times after the injection of rat serum albumin, trace-labelled with [³⁵S]-sulphanilic acid, and the spleens were removed. At the same time other organs were taken out and their content of protein-bound ³⁵S and ³⁵S extractable with perchloric acid was determined. These observations have permitted some tentative conclusions to be drawn about the sites of protein catabolism, and they form the subject of this paper.

MATERIALS AND METHODS

The preparation of rat serum albumin, trace-labelled with [³⁵S]sulphanilic acid, is described by Hawkins & Haurowitz (1961). A portion (3.0 mg.) of this preparation was injected intraperitoneally into seven rats, which were killed at various times up to 11 days later. Blood (about 3 ml.) was obtained in an oxalated syringe by cardiac puncture and the plasma separated by centrifuging. Then

the spleen, lungs, liver, kidneys, a sample of muscle from the abdominal wall, and the inguinal lymph nodes, were rapidly excised, weighed and all except the spleens frozen at -15° until they could be used. The spleens were treated immediately as described by Hawkins & Haurowitz (1961). The other tissues were treated similarly. They were thawed, chopped finely with scissors and homogenized with five times their weight of cooled 0.10M-NaCl-0.05M-sodium citrate (saline-citrate; Chargaff, 1955). The homogenates were centrifuged at 10 000g for 20 min. at 0° and the residues re-extracted with saline-citrate (three times the organ weight) and centrifuged again. The extracts were combined and their total volume was measured. The residues were evenly suspended in 5.0 ml. of saline-citrate. A measured portion of each of the tissue extracts and residues and plasma samples was extracted by adding 2 vol. of 10% (w/v) HClO₄, centrifuging and re-extracting the residue twice with 1 vol. of 7% HClO₄. The extracts were combined and made up to a convenient volume for the determination of radioactivity. The precipitated protein was suspended in 5 ml. of water and the protein-bound ³⁵S in this fraction determined by the methanol-acetate procedure (Hawkins & Haurowitz, 1961).

Determination of radioactivity. Protein-bound ³⁵S was measured by weighing samples (30±0.2 mg.) of the dried and finely powdered protein on nickel-plated planchets and counting in a windowless Geiger counter (Banks, Blow & Francis, 1956). ³⁵S in the HClO₄ extracts was determined by Steinberg's (1959) method. Portions (3.0 ml.) of the extracts were pipetted on to 1.0 g. of finely ground *p*-terphenyl (L. Light and Co. Ltd.) in a glass dish and counted with a scintillation counter. A drop of the neutral detergent Stergene was added to enable the HClO₄ extract to wet the

hydrophobic *p*-terphenyl. Under the conditions used the background count was about 30 counts/min. Sample counts of less than 10 counts/min. above background have been recorded as not significant. The total radioactivity of the protein injected as determined by this method was about 7×10^6 counts/min.; as determined with the windowless Geiger counter it was slightly lower. To facilitate comparison between radioactivity determinations in the different counters, all counts/min. have been corrected to an arbitrary figure of 10^6 counts/min. injected.

The protein-distribution factor for each organ was calculated as described by Hawkins & Haurowitz (1961). This is equal to (counts/min. protein-bound ^{35}S /g. of tissue)/(counts/min. protein-bound ^{35}S /ml. of plasma). As explained there, this is useful for comparing results from tissues of different weights.

RESULTS AND DISCUSSION

As discussed by Hawkins & Haurowitz (1961), the protein-distribution factor gives a measure of the volume of the extracellular-fluid compartment of an organ, provided that all the radioactive protein stays in the extracellular fluid. In general, the protein-distribution factors of the tissues investigated tended to increase as the time after injection increased, so the values of this factor observed 2 days after injection may give the volume of the extracellular-fluid compartment, or they may overestimate it. There are several reasons why the protein-distribution factor may have a high value. In the present work, the existence of a high value has been taken to mean that the protein in the tissue has passed out of the extracellular-fluid compartment in some way. This is a very likely prerequisite for catabolism to take place, but it does not prove that the tissue in question can catabolize the protein.

Only very small amounts of non-protein-bound ^{35}S were found in the plasma 5 days and more after injection (see Table 1). Thus any appreciable amount of non-protein-bound ^{35}S found in the saline-citrate extract of a particular tissue at these times cannot have come solely from the plasma trapped in the tissue, but was probably a product of catabolism of the injected protein by that tissue.

In the present work perchloric acid has been used to precipitate protein and to extract soluble degradation products. Any method of protein precipitation must be somewhat arbitrary since a search is being made for degradation products of a protein and the larger ones will probably have properties very similar to those of the injected protein. Treatment with perchloric acid is a widely used procedure for precipitating proteins and separating them from non-protein material, but the upper size limit (if indeed size is the main determining factor) of peptides derived from degraded proteins that will still be extracted by perchloric acid is

unknown. It is quite likely therefore that extraction with perchloric acid may slightly under-estimate the degree of degradation of protein, but it must give some indication of the amount of low-molecular-weight material produced in this process.

Plasma. Table 1 shows that there was some ^{35}S extractable from the plasma by perchloric acid 2 days after injection. This probably consists of breakdown products of the injected protein which have diffused out of various organs where catabolism has taken place. At later times the amount of this perchloric acid-extractable ^{35}S decreased until there was none in the plasma. It would be expected that catabolism would proceed at a higher rate at short times after injection when there was a relatively high concentration of the injected protein in the body. The protein-bound ^{35}S disappeared from the plasma at an exponential rate, with a half-life of 1.9 days. Weigle & Dixon (1957) reported that injected rabbit serum albumin disappeared very rapidly from the blood of rats.

Spleen. As this organ was required for other purposes, it was not possible to extract the saline-citrate extracts with perchloric acid. The protein-bound ^{35}S in saline-citrate extracts of this organ was therefore determined by precipitation with methanol-acetate. Table 2 shows that considerable amounts of protein-bound ^{35}S had penetrated out of the extracellular-fluid compartment by 5 days after injection. At all times after injection only about half the protein-bound ^{35}S in the organ was extractable with saline-citrate. The protein-bound ^{35}S insoluble in saline-citrate is probably in the form of fairly firm complexes between the rat serum albumin and some tissue components. As the absolute amount of ^{35}S insoluble in saline-citrate decreases with time, it is possible that these complexes represent an intermediate stage in the catabolism of the protein. The occurrence of considerable amounts of ^{35}S extractable by perchloric acid from the insoluble residue strongly suggests that catabolism of the protein occurs in the spleen.

Table 1. *Radioactivity of rat plasma after injection of rat serum albumin trace-labelled with ^{35}S*

All counts/min. are corrected to 10^6 counts/min. injected.

Time after injection (days)	Protein-bound ^{35}S (counts/min./ml.)	^{35}S extracted by HClO_4 (counts/min./ml.)
2	9930	238
2	9444	226
5	3175	88
5	3510	76
8	685	34
11	408	ns*
11	317	ns

* Not significant; less than 10 counts/min. above background.

Table 2. *Radioactivity of spleen fractions after injection of rat serum albumin trace-labelled with ^{35}S* All counts/min. are corrected to 10^6 counts/min. injected.

Time after injection (days)	Wt. of spleen (g.)	Protein-bound ^{35}S in		Protein-distribution factor*	^{35}S extracted from residue by HClO_4 (counts/min.)
		Saline-citrate extract (counts/min.)	Residue (counts/min.)		
2	1.26	1385	1110	0.26	1160
2	0.61	1052	972	0.35	760
5	0.92	837	1032	0.64	730
5	0.89	909	1248	0.66	780
8	1.97	547	699	0.92	300
11	0.89	628	645	3.51	160
11	0.86	408	405	2.98	230

* See Materials and Methods section.

Table 3. *Radioactivity of tissue fractions after injection of rat serum albumin trace-labelled with ^{35}S* All counts/min. are corrected to 10^6 counts/min. injected.

Time after injection (days)	Wt. of tissue (g.)	Protein-bound ^{35}S in		Protein- distribution factor*	^{35}S extracted by HClO_4 from	
		Saline-citrate extract (counts/min.)	Residue (counts/min.)		Saline-citrate extract (counts/min.)	Residue (counts/min.)
Liver						
2	10.2	11 350	1960	0.13	14 900	2955
2	8.1	10 120	1700	0.15	12 900	1040
5	9.4	6 270	1346	0.25	5 590	1010
5	9.1	5 650	1320	0.22	6 460	675
8	17.2	1 828	735	0.22	940	0
11	11.8	3 430	1025	0.92	2 490	500
11	10.4	1 800	763	0.78	1 830	380
Kidney						
2	1.86	4 980	705	0.30	9 195	410
2	1.87	5 130	972	0.34	10 170	1120
5	1.92	3 660	777	0.73	7 870	620
5	2.00	4 030	846	0.70	7 250	750
8	3.03	2 260	498	1.33	3 050	200
11	2.32	2 835	927	3.97	2 960	500
11	2.32	1 523	957	3.37	2 790	370
Lung						
2	1.20	2 645	93	0.23	230	0
2	1.11	2 260	201	0.23	280	0
5	1.49	1 326	128	0.31	456	ns†
5	1.34	993	66	0.23	180	ns
8	1.58	350	81	0.40	ns	0
11	1.25	154	36	0.37	ns	ns
11	1.30	195	54	0.54	420	ns
Skeletal muscle						
2	1.60	927	234	0.07	266	ns
5	1.93	423	111	0.09	320	ns
5	2.02	403	30	0.06	ns	0
8	1.47	90	27	0.12	ns	0
11	1.84	23	0	0.03	ns	ns
11	1.22	36	17	0.14	ns	ns
Lymph node						
2	0.85	1 665	60	0.20	445	0
2	0.30	662	20	0.24	162	0
5	0.77	742	30	0.32	277	0
5	0.62	604	25	0.29	191	0
8	1.06	287	0	0.39	200	ns
11	0.64	95	0	0.36	128	ns
11	0.78	166	0	0.67	187	ns

* See Materials and Methods section.

† Not significant (less than 10 counts/min. above background).

Liver. As might be expected from its large size and high metabolic activity, large amounts of ^{35}S were found in this organ (Table 3). Since the proportion of protein-bound ^{35}S insoluble in saline-citrate was comparatively low, complexes between the protein and tissue components are not likely to be present to any great extent in this organ. This is also suggested by the figures for the protein-distribution factor, which were much lower than those for the spleen, despite the fact that both organs have sinusoidal vascular systems and are presumably fairly rich in blood. However, there is definite evidence that some protein-bound ^{35}S had left the extracellular-fluid compartment 11 days after injection, and this may possibly have happened earlier. Breakdown products from the injected protein were extractable by perchloric acid from the saline-citrate extracts in considerable quantities throughout the period of observation and small amounts were also usually extractable from the insoluble residue. These findings support the claim of Cohen & Gordon (1958) that the liver catabolizes serum albumin.

Kidney. Next to the liver, this organ contained the greatest amount of protein-bound ^{35}S of any organ studied (Table 3), and the concentration of ^{35}S was a good deal higher than in the liver. All of the protein-breakdown products formed in the animal have to pass through the kidney if they are excreted in the urine, but this would hardly account for the large amount of protein-bound ^{35}S , most of which would be in a form not likely to be excreted by this route. The high values of the protein-distribution factor suggest that a good deal of protein-bound ^{35}S has left the extracellular-fluid compartment. Compared with the spleen, only a small proportion of this protein-bound ^{35}S appears to have formed complexes with insoluble tissue constituents, since comparatively little protein-bound ^{35}S was unextractable by saline-citrate. The large amounts of ^{35}S extractable by perchloric acid from the saline-citrate extracts could be breakdown products of the protein, arising by catabolism elsewhere, or they could have arisen by degradation of the protein in the kidney. Because of the large amount of protein-bound ^{35}S present, it seems likely that some of the extractable ^{35}S is in the latter class and therefore that this organ is a site of catabolism of serum albumin.

Lungs. Most of the protein-bound ^{35}S of the lungs was extractable by saline-citrate throughout the experiment (Table 3). The low values for the protein-distribution factor, which only began to rise appreciably at 8 days, suggest that little protein passed out of the extracellular-fluid compartment. Only small amounts of ^{35}S were extractable by perchloric acid from the saline-citrate extracts, so that it is concluded that this organ can cata-

bolize serum albumin only to a limited extent, if at all.

Skeletal muscle. As shown in Table 3, most of the values for the protein-distribution factor were low. It is not known whether this is due to a low content of extracellular fluid in muscle or to slow equilibration between this extracellular fluid and the plasma. The higher values observed in two cases may have been due to slight contamination with blood, even though the pieces of tissue were carefully blotted to remove adherent blood immediately after removal from the animal. The protein-bound ^{35}S in the tissue was nearly all extractable by saline-citrate, and the small amounts of ^{35}S extractable by perchloric acid from the saline-citrate extracts of two pieces of muscle were on the lower limit of detection. As might be expected from its highly specialized function, catabolic activity of this tissue towards serum albumin was not demonstrable.

Lymph nodes. Lymphatic tissue was included in this survey since it may well play a part in the degradation of heterologous proteins, aided perhaps by the immune response of the host animal. When the lymph nodes were removed from the animals no attempt was made to free them completely from the fat in which they were embedded. After homogenization and centrifuging, the fat floated to the top of the tube as a solid white mass, which was removed, weighed and the weight subtracted from the total weight of the tissue. The weights in Table 3 therefore refer to the weights of the nodes themselves.

The lymph nodes seem to possess only very limited catabolic activity with respect to homologous serum albumin, judging by the very small extent to which protein-bound ^{35}S passed out of the extracellular-fluid compartment. Barely detectable amounts of protein-bound ^{35}S were unextractable by saline-citrate and there were only small quantities of ^{35}S extracted by perchloric acid from the saline-citrate extracts.

SUMMARY

1. Plasma, spleen, liver, kidneys, lungs, lymph nodes and samples of skeletal muscle from rats have been analysed for protein-bound ^{35}S and ^{35}S extractable with perchloric acid at various times after intraperitoneal injection of rat serum albumin, trace-labelled with ^{35}S .

2. From the observed distribution of protein-bound ^{35}S and ^{35}S extractable with perchloric acid it is suggested that spleen, liver and kidneys all catabolize homologous serum albumin. Lungs and lymph nodes may exhibit very limited catabolic activity, whereas skeletal muscle has no such activity.

The author is very grateful to Professor A. Wormal, F.R.S., for his encouragement, and to Miss A. Routledge for her valued technical assistance. The work was supported by a grant to St Bartholomew's Hospital from the British Empire Cancer Campaign.

REFERENCES

- Banks, T. E., Blow, L. W. & Francis, G. E. (1956). *Biochem. J.* **64**, 408.
- Chargaff, E. (1955). In *The Nucleic Acids*, vol. 1, p. 307. Ed. by Chargaff, E. & Davidson, J. N. New York: Academic Press Inc.
- Cohen, S. & Gordon, A. H. (1958). *Biochem. J.* **70**, 544.
- Hawkins, J. D. & Haurowitz, F. (1961). *Biochem. J.* **80**, 200.
- Steinberg, D. (1959). *Nature, Lond.*, **183**, 1253.
- Weigle, W. O. & Dixon, F. J. (1957). *J. Immunol.* **79**, 24.

Biochem. J. (1961) **80**, 214

Uptake of Pyriethamine by Tissue of Rats

BY G. RINDI AND V. PERRI

Institute of Human Physiology, University of Pavia, Italy

(Received 13 December 1960)

Although the thiamine and thiamine diphosphate content of the tissues of animals treated with pyriethamine, 1-[(4-amino-2-methylpyrimidin-5-ylmethyl)-2-methyl-3-(β -hydroxyethyl)pyrimidin-ium bromide hydrobromide, has been studied, no data are available regarding the pyriethamine content that may be related to thiamine levels, and that might clarify the mechanism producing avitaminosis B₁ in animals treated with pyriethamine. Administration of pyriethamine causes a decrease of the total thiamine and thiamine diphosphate content of all the tissues, particularly of the brain, in mice and rats (De Caro, Rindi & Grana, 1954; De Caro, Rindi, Perri & Ferrari, 1956*a, b*, 1958; De Caro, Rindi & Ferrari, 1958; Koedam & Steyn-Parvè, 1959), and in pigeons (Koedam, Steyn-Parvè & van Rheeën, 1956; Koedam, 1958). The diminution in the brain is both marked and early, whereas in the alimentary thiamine deficiency the brain more firmly preserves its thiamine content (Salcedo, Najjar, Holt & Hultzer, 1948; Rindi, Ferrari & Perri, 1954).

The present paper describes a microfluorimetric method for determining pyriethamine in rat tissues and reports the results obtained in animals treated with pyriethamine, with and without thiamine.

EXPERIMENTAL

Materials. Adsorbent charcoal was prepared by treatment of activated charcoal (50SL, produced by Italian C.E.C.A., Milan) with cholesteryl stearate (British Drug Houses Ltd.), as described by Siliprandi & Siliprandi (1954). Columns were prepared according to Rindi & de Giuseppe (1961). Amberlite IRC-50 resin (British Drug Houses Ltd., CG-50) was utilized in a carboxylic form after treatment with 2*N*-HCl, followed by washing with

water until disappearance of the Cl⁻ reaction. Pyriethamine was a commercial sample of Neopyriethamine hydrobromide purchased from the California Corp. for Biochemical Research, Los Angeles 63, Calif., U.S.A. The mono- and di-phosphate were prepared by the method of Viscontini, Bonetti & Karrer (1951) and purified on ion-exchange resin. Cellulose powder (Whatman, ash-free cellulose) was washed with 50% (v/v) ethanol, filtered on a Buchner funnel, thoroughly rinsed with water and dried.

Determination of pyriethamine. The rapidly excised tissue (liver, brain, kidney, 3–5 g.; muscle, 9–11 g.) was weighed and homogenized in 2.5 vol. of 0.5*M*-HClO₄ in a MSE homogenizer. After centrifuging (3500 *g*), the clear supernatant fluid was separated and the residue again homogenized in 1 vol. of 0.5*M*-HClO₄ and centrifuged. The combined supernatant fluids were neutralized (pH 6.8–6.9) with 4*M*-KOH. After 20 min. at 0°, the precipitated KClO₄ was removed by centrifuging in a refrigerated centrifuge. To the clear extract, at room temperature and adjusted to pH 4.5 with 50% (v/v) acetic acid, 1 ml. of a freshly prepared aqueous suspension (100 mg./ml.) of Taka-diastase (Parke Davis and Co. Ltd.) was added. The mixture was incubated overnight at 37°. The solution, adjusted to pH 6.8–6.9 with NaOH, was then passed through a column (1.2 cm. diam.) of charcoal-cellulose powder (1:5); 200 mg. of charcoal for liver, brain and kidney, 300 mg. for muscle. The flow rate was 6–8 drops/min. The column was washed with 30 ml. of water and eluted with 25 ml. of 10% (v/v) propan-1-ol (Rindi & de Giuseppe, 1961). The eluate was passed through a column (3 cm. × 0.9 cm.) of Amberlite IRC-50. The flow rate was 10–12 drops/min. The column was washed with 20 ml. of water and eluted with about 25 ml. of 0.1*N*-HCl. The eluate was made up to 25 ml. with the eluent, if necessary. Solid KCl (400 mg.) was dissolved in each of two samples of 1–2 ml. of eluate, adjusted to 2 ml. with water, in stoppered centrifuge tubes; then 0.5 ml. of 10*N*-NaOH was added to each. The samples were incubated for 60 min. at 37° to destroy the thiamine and 0.5 ml. of 10*N*-NaOH was added to one tube (B) and 0.5 ml. of 0.20% (w/v) K₃Fe(CN)₆ in