- Giri, K. V., Narasimha Rao, P. L., Saroja, K. & Venkataraman, R. (1953). *Naturwissenschaften*, **40**, 484.
- Giri, K. V. & Nigam, V. N. (1953). *Naturwissenschaften*, **40**, 343.
- Giri, K. V. & Nigam, V. N. (1954). *J. Indian Inst. Sci.* **36**, 49.
- Giri, K. V., Saroja, K., Venkataraman, R. & Narasimha Rao, P. L. (1954). *Arch. Biochem. Biophys.* **51**, 62.
- Halsall, T. G., Hirst, E. L. & Jones, J. K. N. (1947). *J. chem. Soc.* p. 1427.
- Hassid, W. Z. & Doudoroff, M. (1950). *Advanc. Enzymol.* **10**, 123.
- Hehre, E. J. (1949). *J. biol. Chem.* **177**, 267.
- Hehre, E. J. (1951). *J. biol. Chem.* **192**, 161.
- Hirst, E. L., Hough, L. & Jones, J. K. N. (1949). *J. chem. Soc.* p. 928.
- Huebner, C. F., Ames, S. R. & Bubl, E. C. (1946). *J. Amer. chem. Soc.* **68**, 1621.
- Jeanes, A., Wilham, C. A., Jones, R. W., Tsuchiya, H. M. & Rist, C. E. (1953). *J. Amer. chem. Soc.* **75**, 5911.
- Norberg, E. & French, D. (1950). *J. Amer. chem. Soc.* **72**, 1202.
- Nussenbaum, S. & Hassid, W. Z. (1951). *J. biol. Chem.* **190**, 673.
- Olson, B. H. & Johnson, M. J. (1949). *J. Bact.* **57**, 235.
- Pan, S. C., Andreasen, A. A. & Kolachov, P. (1950). *Science*, **112**, 115.
- Pan, S. C., Nicholson, L. W. & Kolachov, P. (1951). *J. Amer. chem. Soc.* **73**, 2547.
- Pan, S. C., Nicholson, L. W. & Kolachov, P. (1953). *Arch. Biochem. Biophys.* **42**, 421.
- Pazur, J. H. & French, D. (1951). *J. Amer. chem. Soc.* **73**, 3536.
- Pazur, J. H. & French, D. (1952). *J. biol. Chem.* **196**, 265.
- Ram, J. Sri & Giri, K. V. (1952). *Arch. Biochem. Biophys.* **38**, 231.
- Reeves, R. E. (1941). *J. Amer. chem. Soc.* **63**, 1476.
- Torriani, A. M. & Monod, J. (1949). *C.R. Acad. Sci., Paris*, **228**, 718.
- Whistler, R. L. & Durso, D. F. (1950). *J. Amer. chem. Soc.* **72**, 677.
- Wolfom, M. L., Thompson, A., O'Neill, A. N. & Galkowski, T. T. (1952). *J. Amer. chem. Soc.* **74**, 1062.

The N-Terminal Groups of Salmine

By D. M. P. PHILLIPS*

Department of Biochemistry, John Curtin School of Medical Research, Australian National University, Canberra, A.C.T., Australia

(Received 18 November 1954)

While measuring the absorption spectra in the range 230–450 m μ . of a series of 2:4-dinitrophenyl-amino acids (subsequently referred to as DNP-amino acids), it was found that DNP-L-proline and DNP-L-hydroxyproline had spectra quite unlike

those of the derivatives of other amino acids found in proteins. This had been noted independently by Schroeder, Honnen & Green (1953) and by Rao & Sober (1954). To illustrate this, Fig. 1 shows the spectrum of DNP-proline (those of DNP-hydroxyproline, DNP-proline methyl ester and DNP-sarcosine are similar) and, for comparison, the spectrum of DNP-threonine. Porter & Sanger (1948), using 1-fluoro-2:4-dinitrobenzene, found that proline was the *N*-terminal residue of salmine, but owing to the extreme instability of DNP-proline in hot acid a quantitative estimation was not possible.

The spectral findings above therefore suggested a new way of proving, from the shape of the spectrum of DNP-salmine, that proline is the *N*-terminal amino acid in the protamine. Moreover, since no hydrolysis is necessary, and as it is reported (Tristram, 1949) that there are no lysine, histidine, tyrosine or cystine residues in salmine, it is reasonable to assume a simple open-chain peptide structure, in which case the spectrophotometric estimation of the proportion of *N*-terminal amino acid should also give the molecular weight.

This paper describes experiments undertaken to throw light on these two problems.

MATERIALS AND METHODS

General. Salmine sulphate from keta salmon (British Drug Houses Ltd.) was used. As far as could be ascertained, the salmine came from mature *Oncorhynchus keta* caught in

Fig. 1. The absorption spectra of DNP-L-proline and DNP-L-threonine. Both solutions 31.7×10^{-6} M in 0.1 N-HCl.

* Present address: The Chester Beatty Research Institute, Fulham Road, London, S.W. 3.

the Fraser River, British Columbia, in late August or September.

Spectra were measured in a Hilger ultraviolet spectrophotometer using 1 cm. silica cuvettes and slit widths corresponding to a band width of 5 Å. If the extinction of a solution exceeded 0.8 (corresponding approximately to 5×10^{-6} M DNP-amino acid), it was diluted in order to be within the limit set by Sanger (1949*a*) for conformity with Beer's Law.

N was determined by a modification (Ma & Zuazaga, 1942) of the micro-Kjeldahl method and P by the micro-method of Berenblum & Chain (1938).

The DNP-amino acids were prepared by the general method of Sanger (1945). DNP-L-proline methyl ester was prepared by the action of diazomethane in ether on an ether suspension of recrystallized DNP-L-proline as described for some other esters by Fletcher, Lowther & Reith (1954). It was free of DNP-proline and 2,4-dinitrophenol. The preparation of DNP-salmine is given under Results.

Light stability of DNP-acids. It was found that acid or alkaline solutions of DNP-proline were entirely bleached by 20 min. exposure to sunlight, and neither free proline nor 2,4-dinitrophenol were detected in the products. Fortunately, DNP-proline methyl ester and DNP-salmine were more stable and were only partly degraded by 10 days' exposure. As a precaution, however, all samples of DNP-derivatives were stored in the dark when not in use.

Purification of the salmine. The salmine sulphate contained 0.4 µg. P/mg., and in water gave a spectrum with a pronounced step (in alkali) or peak (in acid) at 270 mµ. (see Fig. 2). This contaminant passed through cellophan along with salmine when ultrafiltered under pressure, so that it was perhaps a nucleotide.

Fig. 2. The absorption spectrum of salmine: (a) before and (b) after chromatography on Dowex-2 OH^- resin. Concentration, 2.15 mg. salmine/ml. in 0.1 N-HCl.

The contaminant could be removed by chromatography. For example, 2.4 g. of salmine sulphate was dissolved in 13 ml. of 0.1 N-HCl and applied to a 2.7×33 cm. column of freshly regenerated Dowex-2 OH^- -form resin (200–400 mesh/in. Dow Chemical Co., Midland, Michigan, U.S.A.)

and the column developed with water. The free salmine came through rapidly and could be detected in the fractions (about 4 ml. each) by the Sakaguchi reaction on spots applied to paper, or merely by the pH, since the salmine fractions were alkaline.

The spectrum of each fraction was examined and satisfactory fractions combined and neutralized with HCl (not H_2SO_4 , as the sulphate is far less soluble). Fig. 2 shows the spectrum of keta salmine before and after Dowex-2 treatment, the concentrations in both cases being 2.15 mg. salmine/ml. in 0.1 N-HCl. The purified salmine had $[\alpha]_D^{25} - 78 \pm 2^\circ$ in water at pH 7 (*c*, 3.01).

For some experiments DNP-salmine free of salt (resulting from the acidification of the bicarbonate used during the coupling with fluorodinitrobenzene) was obtained by precipitating with 2.3 M trichloroacetic acid to a final acid concentration of 0.4 M. This precipitated 80–90% of the DNP-salmine as an orange oil, which was then washed with ether.

Nitrogen content of the salmine. To relate the amount of terminal DNP-amino acid to the amount of protamine present required a knowledge of the N content of the free protamine. This was measured by preparing the free base by chromatography through Dowex-2 OH^- resin as described above and immediately placing a known volume of the alkaline solution in a weighing bottle in a desiccator over KOH. This took the solution to dryness without the formation of salmine carbonate, and the sample was then weighed. Another part of the solution was diluted and total N was determined in sextuplicate. These measurements gave 30.5–30.9%, mean 30.7% N in the salmine treated with Dowex-2.

Paper chromatography. For two-dimensional chromatograms of amino acids the solvent systems: *n*-butanol-acetic acid-water (5:1:4, by vol.) and phenol-water were used. For DNP-amino acids satisfactory two-dimensional chromatograms were obtained by running first with *n*-butanol saturated with a small excess of 0.017 N acetic acid and then with the butanol-acetic acid solvent mentioned above or with 1.2 M sodium phosphate buffer (pH 6.5) (modified from Levy, 1954). No. 1 Whatman paper was used throughout.

RESULTS

The spectrum of the purified salmine described above showed that it was free of nucleic acid, tyrosine and tryptophan. An immediate effect of the purification was that chromatograms of hydrolysates of the salmine now showed arginine as a large symmetrical spot, whereas previously under all conditions of hydrolysis tried it had the appearance of two equal spots in contact, one being slightly faster in both butanol-acetic acid and phenol.

The reaction of salmine with 1-fluoro-2:4-dinitrobenzene (FDNB)

Salmine (241 mg.) in 10 ml. neutral solution as the hydrochloride in water was mixed with 2 ml. M- NaHCO_3 , 13 ml. ethanol and 0.3 ml. FDNB in a weighed 50 ml. flask with a long neck and glass stopper. During the shaking period this reaction mixture warmed up to 27° and formed a clear solution. All sampling was done by weight. After weighing the reaction flask a sample of 2.0 ml. was

pipetted into a weighed test tube fitted with a glass stopper, and the reaction flask then weighed again. The total weight removed (sample + wetting on the pipette) enabled the amount of salmine still left in the reaction flask to be calculated. The sample tube was now weighed again to give the salmine in the sample itself. The sample was now mixed with 1 ml. of water and 7 ml. of peroxide-free ether, shaken thoroughly, and the ether extract discarded. This was repeated twice and the aqueous layer then acidified with 3 drops of 11*N*-HCl and extracted with ether until the extracts gave no colour (due to dinitrophenol) when shaken with alkali. This procedure removed all the ethanol, FDNB and dinitrophenol. The remaining aqueous layer was freed from ether by standing in the dark under a jet of filtered air for an hour and was then diluted to

Fig. 3. The reaction of salmine with fluorodinitrobenzene. Spectra of samples taken at the times indicated. Curve *A* is the difference: 75 hr. sample - 4.5 hr. sample. Average concentration, 0.095 mg. salmine/ml. 0.1 *N*-HCl (range 0.094-0.096 mg./ml.).

10.0 ml. with water. For the optical measurements 0.50 or 1.00 ml. of this solution was diluted to 10.0 ml. with 0.1 *N*-HCl and the spectrum from 230 to 420 *mμ*. read every 10 *mμ*. or every 2.5 *mμ*. at inflexions. Fig. 3 shows the spectra of samples taken after 1, 2.67, 4.5, 7.5 and 75 hr. reaction, and Fig. 4 the development of optical density at the peak (370 *mμ*.) and at the trough (*ca.* 300 *mμ*.) as the reaction proceeded.

The spectra in Fig. 3 from 250 to 420 *mμ*. have the character of the spectrum of DNP-proline, since the

peak at 370 *mμ*. is at a longer wavelength than usual for DNP-amino acids or peptides, the trough at 300 *mμ*. is large and smooth, and there is no inflexion at 390 *mμ*. As the salmine was free of hydroxyproline, the indication is that the *N*-terminal amino acid in salmine is proline. The upper curve in Fig. 4 shows that the reaction forming the DNP-prolyl group was complete in 4.5 hr., and shaking for a further 3 days did not alter the value at 370 *mμ*. The lower curve in Fig. 4, however, shows that there was a slow reaction (the 'trough material' mentioned below) which was not complete even in 3 days. Fortunately, however, it contributed less than 2% to the optical density at 370 *mμ*.

The molecular weight of salmine could not be calculated directly from the optical density at 370 *mμ*., however, because of the presence of small amounts of other DNP-amino acids as shown by the maxima (in Fig. 3) being at 370 *mμ*. rather than 375 *mμ*. (the maximum for DNP-proline ester), and by the slight inflexion in the DNP-salmine spectrum from 250 to 300 *mμ*. when compared with DNP-proline ester. As the size of this inflexion did not change after 4.5 hr. reaction, it was not connected

Fig. 4. The reaction of salmine with fluorodinitrobenzene. The changes in optical density at the 370 *mμ*. peak and the 295-310 *mμ*. trough as reaction proceeds. Average concentration: 0.095 mg. salmine/ml. 0.1 *N*-HCl (range 0.094-0.096 mg./ml.).

with the problematical 'trough material' mentioned above. The correction to be applied on account of these other DNP-acids has been estimated as follows:

(a) The DNP-amino acids bearing a single DNP-amino group (including *N*^ε-DNP-ornithine) give spectra in acid like that of DNP-threonine, shown in Fig. 1. If it is assumed that the spectrum of DNP-salmine (making due allowance for the absorption of salmine itself, shown in Fig. 2*b*) should be like that of DNP-proline ester, then the 250-300 *mμ*.

inflexion could be accounted for by the presence of approx. one DNP-amino group for every 6 DNP-prolyl (DNP-amino) groups, i.e., by approx. 15% of DNP-imino groups. This was assessed by calculations in which the optical densities of a standard solution of DNP-threonine were added to those of a standard solution of DNP-proline ester to produce an inflexion in its spectrum of the size observed in DNP-salmine. A similar result was given by subtracting the optical densities of DNP-threonine in

products on paper. The individual spots were estimated, after excision and elution with 4.0 ml. of 40% acetone-60% 0.1N-HCl (v/v), by measurement of the optical densities at their maxima. N^6 -DNP-ornithine, DNP-arginine, DNP-serine, DNP-glycine and traces of DNP-alanine and DNP-(leucine or isoleucine) were found as well as some artifacts. An example of the chromatograms is shown in Fig. 5. By extrapolation of the estimations of each DNP-amino acid back to zero hydrolysis duration, the hydrolytic loss of the DNP-amino acid was accounted for.

Fig. 5. Composite chromatogram of DNP-salmine hydrolysates: I_1 and I_2 , 'indicator' spots, yellow in NH_3 , colourless in acid, not ether-soluble; P_1 and P_2 , DNP-peptides, present only in 1 hr. hydrolysates; Orn, N^6 -DNP-ornithine, giving positive ninhydrin test and distinguished from N^6 -DNP-lysine by running in *tert*-amyl alcohol saturated with 0.1M sodium phthalate buffer (pH 6.0) on similarly buffered paper (Blackburn & Lowther, 1951); Arg, DNP-arginine, giving positive Sakaguchi test (a DNP-prolyl peptide is also present here in 1-3 hr. hydrolysates); Ser, DNP-serine + DNP-glycine; DNP, 2:4-dinitrophenol (with DNP-proline in 1 and 2 hr. hydrolysates); Ala, DNP-alanine, L, DNP-(leucine or isoleucine); DNA, 2:4-dinitroaniline, giving an impermanent red colour with alkaline hypobromite. Solvent 1, *n*-butanol to which 0.017N acetic acid was added to saturation. Solvent 2, *n*-butanol-acetic acid-water (5:1:4, by vol.).

a similar manner from those of DNP-salmine. In this case a still smoother spectrum, like that of DNP-proline ester, was produced. Correction by this method, however, is uncertain because the spectrum of a pure DNP-proline-terminated salmine is unknown. (b) Another estimate of the correction was provided by measurement of the DNP-amino acids other than proline. This was done by hydrolysing in quadruplicate DNP-salmine samples (each equivalent to 5.8 mg. salmine) in 6N-HCl at 120° for 3, 6, 12, 18 and 24 hr. and chromatographing the

The molecular extinction coefficient of DNP-salmine

The molecular extinction coefficients (ϵ) of the DNP-amino acids at their maxima (at ca. 360 $m\mu$.) are not all the same, and calculation of the molecular weight of DNP-salmine from its optical density required a value of ϵ for DNP-proline in peptide form. In order to overcome some of the uncertainty about the precise value of ϵ to be used, attempts were made to measure the amount of DNP-groups by reduction with titanous chloride (Mills, 1952), but since quantitative results could not be obtained with DNP-proline itself, this was not pursued further. Sanger (1949a) and Fletcher *et al.* (1954) have shown that in the spectra of the peptides DNP-phenylalanylvaline and DNP-glycylglycine, the main peaks had lower values of ϵ_{max} at a shorter wavelength than DNP-phenylalanine and DNP-glycine respectively. No proline peptide was available, so DNP-L-proline methyl ester was used. Standard solutions were made by diluting the ester in methanol with a large excess of 0.1N-HCl or 0.2M sodium borate buffer (pH 8.5), so that the final methanol content did not exceed 1% (v/v).

In these solvents the ester had $\epsilon_{1cm}^{375m\mu} = 17450$, and $\epsilon = 17400$ at 370 $m\mu$., the wavelength of the absorption peak in the DNP-salmine spectrum. This latter value was used, with corrections mentioned below, in the calculation of the molecular weight of salmine.

A change in the spectrum of a DNP-amino acid similar to that described above (on incorporation in a peptide or esterification) is produced when an alkaline solution is acidified. Thus, DNP-L-proline had $\epsilon_{365m\mu}^{365m\mu} = 19000$ average in pH 8.5 borate buffer, and $\epsilon_{375m\mu}^{375m\mu} = 17400$ average in 0.1N-HCl.

As described in the previous section, there were small amounts of *N*-terminal amino acids other than proline in the salmine. In the calculation of the molecular weight, therefore, corrections have been made (a) for the N^6 -DNP-ornithine which is not terminal and (b) for the DNP-arginine, serine, etc., which have lower ϵ than DNP-proline. At 370 $m\mu$., the wavelength of the maximum in the DNP-salmine spectrum, N^6 -DNP-ornithine had $\epsilon = 15500$,

Table 1. *Calculation of proportions of N-terminal residues of salmine*

Average salmine content of 4.5, 7.5, 16, 25, 49 and 75 hr. samples: 0.095 mg./ml.

Average extinction, $E_{1\text{ cm.}}^{370\text{ m}\mu}$, of these samples: 0.440.*

Corrections: N^{δ} -DNP-ornithine, DNP-arginine, (DNP-serine + DNP-glycine) and (DNP-alanine + DNP-leucine or iso-leucine): found in 0.095 mg. salmine: 1.6, 1.6, 1.0 and 1 (approx.) $\times 10^{-9}$ moles respectively.

In 1 ml. 0.1N-HCl these have $E_{1\text{ cm.}}^{370\text{ m}\mu}$: 0.025, 0.021, 0.013 and 0.013 respectively.

$E_{1\text{ cm.}}^{370\text{ m}\mu}$ of DNP-salmine after deducting non-terminal ornithine: 0.415.

$E_{1\text{ cm.}}^{370\text{ m}\mu}$ due to DNP-arginine, etc.: 0.047; equivalent to: 3.6×10^{-9} moles/ml.

Whence $E_{1\text{ cm.}}^{370\text{ m}\mu}$ due to DNP-proline: 0.368; equivalent to: 21.2×10^{-9} moles/ml.

Total N-terminal groups: 24.8×10^{-9} moles/ml., containing 0.095 mg. salmine.

Hence there is 1 mole terminal group in 3830 g. salmine, or 1 mole terminal proline in 4480 g. of salmine, or, including ornithine, 1 mole DNP-group in 3600 g. of salmine.

DNP-groups other than proline amount to 20% (mol. prop.) of all DNP-groups (estimated from shape of the spectra (p. 405) about 15%).

* Two other preparations of DNP-salmine gave extinctions of 0.448 and 0.458 for 0.095 mg. salmine/ml. in 0.1N-HCl.

and the other acids an average $\epsilon = 13150$, all in 0.1N-HCl. These values, together with $\epsilon = 17400$ for DNP-proline ester, were accordingly used as shown in Table 1.

The material filling the trough in the spectrum of DNP-salmine

The reactions producing the peak at 370 m μ . and the inflexion at 265 m μ . in the spectrum of DNP-salmine were complete after 4.5 hr. (see Figs. 3 and 4). The difference between the spectra of the 75 hr. sample and the 4.5 hr. sample should thus give the spectrum of the material resulting from the slow reaction which fills up the trough at 300 m μ . This difference is shown in the lowest curve (A) in Fig. 3. That this effect was not due to extraneous material derived from the Dowex-2 resin used for the purification was shown when unpurified salmine sulphate was coupled with FDNB. In this case too, the trough filled up as reaction proceeded.

The nearest approach to this spectrum among the DNP-amino acids known to occur in proteins is given by *Im*-DNP-histidine and *O*-DNP-tyrosine. A search for these two amino acids on chromatograms of acid hydrolysates of salmine, using the Pauly test, failed to reveal any tyrosine, but a trace of histidine, amounting to less than 0.1% (1 mole in 155 000 g.) was detected. This, however, is far too little to account for the optical densities observed. In terms of *O*-DNP-tyrosine the amount formed after 75 hr. reaction of salmine with FDNB was ca. 0.7 mole/4000 g. salmine. That the material was actually a DNP-derivative was shown by keeping DNP-salmine in pH 8.5 borate buffer at 25°. Under these conditions the extinction decreased at 300 m μ . and increased at 370 m μ . with the formation of dinitrophenol, the process being complete in about 3 days. A slight degradation of the peak at

370 m μ . also occurred under these conditions. It was for this reason that all the samples taken for the spectra in Fig. 3 were made acid as soon as the excess FDNB had been removed by ether, and the spectra then measured in acid solution.

Precipitates from DNP-salmine

Two distinct precipitates have been obtained from DNP-salmine. (1) When DNP-salmine was prepared and the acid solution concentrated *in vacuo* to 30 mg. salmine/ml., a yellow precipitate appeared. This was washed 4 times with 0.1N-HCl and when dried amounted to 0.5–0.8% of the weight of salmine taken. It could be dissolved in 6N-HCl only by heating at 100° for at least 3 hr. The spectrum of this solution had maximum density at 360 m μ . corresponding to 1 mole N^{α} - or N^{β} - or N^{ϵ} -DNP/1500 g., which is about the average value for DNP-proteins (DNP-insulin, 2000 g.; DNP-globin (horse) 1430 g.; see Sanger, 1949b). Hydrolysis for 16 hr. at 120° gave chromatograms showing all the usual protein amino acids except tryptophan, tyrosine and histidine. Lysine was present as N^{ϵ} -DNP-lysine, and there were large spots of arginine, alanine and leucine. Moreover, the glutamic acid spot was larger than the serine, so that this precipitate must represent an extraneous protein sufficiently basic to travel with salmine during the purification process. If the DNP-salmine preparation is not concentrated, this DNP-protein remains in solution, but the quantity present is too small to be considered in correcting the absorption measurements. (2) When solutions of DNP-salmine (0.19–1.9 mg./ml. in borate buffer, pH 8.5 or 9.2) were allowed to stand for 24 hr. or more, a small precipitate appeared, which was readily soluble in acid. Chromatograms of hydrolysates of this material differed from those of whole DNP-salmine hydrolysates in having definite aspartic and glutamic acid

spots and an exceptionally small isoleucine content. The weight of this precipitate was about 2 % of the DNP-salmine taken.

DISCUSSION

Correlation of published data on the chemistry of salmon protamines is complicated by different authors having studied 'salmines' obtained from different genera and species of Salmonidae, which have not always been specified, or even known for certain, by these authors. Several chemical analyses have been made (see Corfield & Robson, 1953, for a summary), and molecular weights were calculated from some of these on the assumption that the amino acids in smallest amounts (alanine and isoleucine) were present to the extent of one residue in each molecule. The analyses of Tristram (1947, 1949) and Corfield & Robson (1953) gave minimum molecular weights of 7460–8260 and 9570–10240 respectively, but apart from the consistency of the analyses there was no evidence that the salmines used were homogeneous. Rauen, Stamm & Felix (1952) and Felix (1953), working with clupeine, have demonstrated its heterogeneity by electrophoretic and ultracentrifugal techniques. The qualitative finding here that a precipitate could be obtained from DNP-salmine containing some aspartic and glutamic acids and poor in isoleucine supports the view that salmine too is a mixture of proteins. Velick & Udenfriend (1951) also found glutamic acid amounting to 0.1 mole/7000 g. of an *Oncorhynchus* salmine. Thus the residues occurring in smallest amount may be those most likely to be variables in the amino acid composition. Whatever the explanation of the differences between the results of Tristram (1947, 1949) and Corfield & Robson (1953), the method used here suggests that the average molecular weight of keta salmine is $3800 \pm 10\%$. This brings the average molecular weight of salmine much nearer the values of 3500 suggested for clupeine (Felix, Fischer, Krekels & Rauen, 1950) and 2700 for sturine (Plaskéev, Yarovenko & Pasynskiĭ, 1945). In addition to the main type of salmine with *N*-terminal proline, accounting for 80 % of the end groups, there are apparently other types of salmine terminating in arginine, serine and glycine. A result using an *N*-terminal method which conflicts with the value given here is that of Velick & Udenfriend (1951). Using their elegant isotopic method employing *p*-iodobenzenesulphonyl chloride (giving 'pipsyl' derivatives) as the coupling agent, they found one mole of terminal proline/6800 g. of *Oncorhynchus* salmine (1.1 moles/7000 g.). This may be a genuine discrepancy due to differences in the salmines, but other partial explanations may be: (a) with insulin (Udenfriend & Velick, 1951) the method gave only half the number of terminal glycine and phenyl-

alanine residues indicated by the FDNB method; (b) the calculation includes the assumption that the added [^{35}S]-pipsylproline was broken down at the same rate on hydrolysis as the [^{131}I]-pipsylproline group in the pipsylsalmine. With DNP-proteins the breakdown of end groups on acid hydrolysis has varied. For example, the breakdown of bis-DNP-lysine was far greater when lysozyme was present (Thompson, 1951), whereas the breakdown of DNP-amino acids in the presence of wool was diminished (Middlebrook, 1951); (c) no search was reported for acid-soluble pipsylamino acids. It might be argued that the end-groups other than proline found here do not originate from salmine but from contaminating protein or degraded salmine. While this cannot be proven without actual isolation of salmine with an arginine, glycine or serine *N*-terminal group, it must be remembered that clupeine was reported as having serine as well as proline end-groups (Felix *et al.* 1950; Šorm & Šormová, 1951), and in addition Ando, Ishii, Hashimoto, Yamasaki & Iwai (1952) reported alanine end-groups.

More recent fractionations of clupeine (Waldschmidt-Leitz, Kühn & Zinnert, 1951; Felix & Krekels, 1953) have given material with *N*-terminal proline groups only. The separated material with *N*-terminal serine residues must, however, still be regarded as clupeine. Likewise all the material examined here must be protamine, since it passed through a Dowex-2 OH^- column along with the bulk of material bearing *N*-terminal proline.

The finding of ornithine (in the form of *N*⁶-DNP-ornithine) in the DNP-salmine (0.064 moles/3800 g.) could be taken as evidence of degradation of a little protamine arginine by alkali. Two considerations, however, argue against this. First, the salmine sulphate was prepared according to Kossel (1928), a method not using alkali. Although the salmine becomes alkaline for a short while during the Dowex-2 purification, it was found that salmine which had not been so treated still contained some ornithine. Secondly, DNP-protamine prepared from a sample of protamine from *Salmo irideus* also showed the presence of *N*⁶-DNP-ornithine in acid hydrolysates.

The method described here has special value for the protamines, which in general form soluble DNP-derivatives and are believed to have only one *N*-terminal group in each molecule. Moreover, the spectrum could be used as a general method to detect and measure the DNP-prolyl group without incurring the considerable degradation caused by acid hydrolysis.

Results similar to those above, but complicated by the presence of a higher proportion of non-proline DNP-groups, have been found for the protamine from *Salmo irideus* (rainbow trout) and will be reported later.

SUMMARY

1. Purified salmine from *Oncorhynchus keta* has been coupled completely with 1-fluoro-2:4-dinitrobenzene and the spectrum of the resulting dinitrophenyl (DNP)-salmine measured in the range 235–420 m μ .

2. The spectrum is similar to the spectrum of DNP-proline.

3. Four-fifths of the *N*-terminal residues in the salmine are proline and the rest mainly arginine, serine and glycine.

4. From the spectrum an average molecular weight of $3800 \pm 10\%$ has been calculated for salmine, assuming all the molecules to be simple open-chain peptides.

5. A secondary slow reaction occurs with fluorodinitrobenzene, which is unexplained.

6. A little *N*⁶-DNP-ornithine was found, which it is thought may be in the native protamine and not a degradation product.

I wish to thank Mr D. A. Maguire for his continued valuable technical assistance and Dr J. A. Broben of the Commonwealth Serum Laboratories, Melbourne, for the gift of a sample of protamine from *Salmo irideus*.

REFERENCES

- Ando, T., Ishii, S., Hashimoto, C., Yamasaki, M. & Iwai, K. (1952). *Bull. chem. Soc. Japan*, **25**, 132.
 Berenblum, I. & Chain, E. (1938). *Biochem. J.* **32**, 295.
 Blackburn, S. & Lowther, A. G. (1951). *Biochem. J.* **48**, 126.
 Corfield, M. C. & Robson, A. (1953). *Biochem. J.* **55**, 517.

- Felix, K. (1953). *The Chemical Structure of Proteins*. (Ed. G. E. W. Wolstenholme and M. P. Cameron), p. 151. London: Churchill.
 Felix, K., Fischer, H., Krekels, A. & Rauen, H. M. (1950). *Hoppe-Seyl. Z.* **286**, 67.
 Felix, K. & Krekels, A. (1953). *Hoppe-Seyl. Z.* **295**, 107.
 Fletcher, C. M., Lowther, A. G. & Reith, W. S. (1954). *Biochem. J.* **56**, 106.
 Kossel, A. (1928). *The Protamines and Histones* (translated by W. V. Thorpe). London: Longmans, Green and Co.
 Levy, A. L. (1954). *Nature, Lond.*, **174**, 126.
 Ma, T. S. & Zuazaga, G. (1942). *Industr. Engng Chem. (Anal.)*, **14**, 280.
 Middlebrook, W. R. (1951). *Biochim. biophys. Acta*, **7**, 547.
 Mills, G. L. (1952). *Biochem. J.* **50**, 707.
 Plaskéev, V., Yarovenko, N. & Pasynskii, A. (1945). *C.R. Acad. Sci., U.R.S.S.*, **49**, 580.
 Porter, R. R. & Sanger, F. (1948). *Biochem. J.* **42**, 287.
 Rao, K. R. & Sober, H. A. (1954). *J. Amer. chem. Soc.* **76**, 1328.
 Rauen, H. M., Stamm, W. & Felix, K. (1952). *Hoppe-Seyl. Z.* **291**, 275.
 Sanger, F. (1945). *Biochem. J.* **39**, 507.
 Sanger, F. (1949a). *Biochem. J.* **45**, 563.
 Sanger, F. (1949b). *Symp. biochem. Soc.* no. 3, p. 21.
 Schroeder, W. A., Honnen, L. R. & Green, F. C. (1953). *Proc. nat. Acad. Sci. Wash.* **39**, 23.
 Šorm, F. & Šormová, Z. (1951). *Coll. Trav. chim. Tchécosl.* **16**, 207.
 Thompson, A. R. (1951). *Nature, Lond.*, **168**, 390.
 Tristram, G. R. (1947). *Nature, Lond.*, **160**, 637.
 Tristram, G. R. (1949). *Advanc. Protein Chem.* **5**, 83.
 Udenfriend, S. & Velick, S. F. (1951). *J. biol. Chem.* **190**, 733.
 Velick, S. F. & Udenfriend, S. (1951). *J. biol. Chem.* **191**, 233.
 Waldschmidt-Leitz, E., Kühn, K. & Zinnert, F. (1951). *Experientia*, **7**, 183.

Quantitative Aspects of Glycine Metabolism in the Rabbit

By OLGA B. HENRIQUES,*† S. B. HENRIQUES* AND A. NEUBERGER

National Institute for Medical Research, London, N.W. 7

(Received 10 December 1954)

Schoenheimer, Ratner & Rittenberg (1939) first observed that the amount of labelled amino acid incorporated into proteins, within a short time after administration of the tagged compound, varied greatly with different tissues. These pioneer experiments were done with rats and the amino acid used was L-leucine which had been labelled with both ¹⁵N and deuterium. Later work (Tarver & Schmidt, 1942; Friedberg, Tarver & Greenberg, 1948; Greenberg & Winnick, 1948; Winnick, Friedberg & Greenberg, 1948), in which carbon- and sulphur-labelled amino acids were used, has amply

confirmed the findings of Schoenheimer *et al.* (1939). The mixed proteins of some tissues such as intestinal tract, liver and kidney were found to contain large amounts of isotopically labelled amino acid within a few hours after administration, while other tissues such as muscle, skin and brain gave only low values, if the comparison was made on a unit weight basis. It was concluded (Schoenheimer & Rittenberg, 1940) that the concentration of isotope found in a particular tissue under the conditions mentioned could be taken as a measure of 'relative chemical activity' of the organ proteins. It has been pointed out (Neuberger, 1952) that this interpretation is based on the assumption that the rate of penetration of the labelled amino acid into the cells of a given tissue is very much greater than the

* Present address: Instituto Butantan, C.P. 65, Sao Paulo, Brazil.

† Fellow of the Brazilian Research Council.