The Digestion and Absorption of Protein by Normal Man

By C. W. CRANE and A. NEUBERGER
Department of Chemical Pathology, St Mary's Hospital Medical School, London, W. 2

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The work to be described in this paper was undertaken as a prelude to investigations on the digestion and absorption of protein in various clinical conditions, particularly those relating to diseases of the intestine. Although a large amount of work has been done on protein absorption in the last 50 years, there is still uncertainty about several important points, such as the nature of the digestion products absorbed by the mucosa or the time required for enzymic hydrolysis and actual absorption (see Fisher, 1954; Nasset, 1957). It appeared to us that ^{15}N-labelled protein might, with advantage, be used in such studies. [^{15}N]-Amino acids, [^{15}N]ammonia and [^{15}N]urea have been used in man, especially by Rittenberg and his colleagues (e.g. Sprinson & Rittenberg, 1949*a*, *b*; San Pietro & Rittenberg, 1953*a*, *b*) and also by Wu (see Wu, 1951; Wu & Snyderman, 1951; Wu & Bishop, 1959; Wu & Sendroy, 1959; Wu, Sendroy & Bishop, 1959), but there are only a few publications on the metabolism of proteins labelled with a stable isotope (White & Parson, 1950; Sharp, Lassen, Shankman, Gebhart & Hazlet, 1956; Sharp, Lassen, Shankman, Hazlet & Kendis, 1957). Protein labelled with ^{131}I has been used in studies on protein digestion in children (Lavik *et al.* 1952).

EXPERIMENTAL

Yeast ^{15}N-labelled protein. A strain of *Saccharomyces cerevisiae* (The Distillers Co. Ltd. no. 568) was harvested from 1 l. of medium made up as follows: glucose (20 g.), $(^{15}NH_4)_2SO_4$ (3·1 g.), KH_2PO_4 (2·5 g.), lactic acid (1·7 ml.), $MgSO_4,7H_2O$ (0·5 g.), $CaCl_2,6H_2O$ (0·25 g.), $FeCl_2$ (1mg.), $ZnSO_4,7H_2O$ (1 mg.), $MnCl_2$ (1 mg.), thiamin (2 mg.), pyridoxine (2 mg.), inositol (20 mg.), nicotinamide (5 mg.), calcium D-pantothenate (4 mg.) and D-biotin (5 μg.). The mixture was brought to pH 5·0 with 2N-NaOH (external indicator) and autoclaved at 15 lb./in.^{2} for 15 min. in a 2 l. conical flask fitted with a sintered-glass air inlet dipping into the medium, and a wide-bore outlet tube. The $(^{15}NH_4)_2SO_4$ was prepared from [^{15}N]ammonium nitrate containing 34·7 atoms % excess of ^{15}N by the method of Schoenheimer & Ratner (1939). A subculture taken from a stock slope of the yeast and incubated for 16 hr. was used for inoculation. A steady stream of filtered air, passed through a sterilized water bubbler, was blown through the medium. The whole apparatus was kept at a constant temperature of 30° and stirred magnetically. After 18 hr. the rate of air flow was increased to a maximum and at intervals of 2 hr. for a further 6 hr. 20 g. of glucose was added and the pH was adjusted to 5·0 (external indicator) with 2N-Na_2CO_3. After leaving it overnight at 30°, the yeast was harvested by centrifuging and washing three times with 250 ml. of water and finally collected on a Büchner filter. Analysis of the supernatant and washings showed that the incorporation of N into the yeast averaged 93·7%. The average yield of wet yeast was 38 g./l. Fragmentation of the yeast was carried out in lots of 7 g. in 50 ml. of water for 2 hr. in a Mullard ultrasonic generator (2 kw), type E 1790, with a working frequency of 15–25 kcyc./sec.; each batch was then poured into three times its volume of chilled ethanol. After centrifuging and washing with cold acetone, followed by ether, the resultant white powder was extracted twice with 5% trichloroacetic acid solution (10 ml. of trichloroacetic acid/g. of wet yeast) for 15 min. at room temperature, followed by a similar extraction at 90° (Dalgliesh & Dutton, 1956) to remove nucleic acids. The residue was washed with a small quantity of water, followed by ethanol and acetone, and finally dried over KOH followed by P_2O_5. Average yield was 3·8 g./l. of medium, with an average content of 90 mg. of N/g. and an average ^{15}N content of 33·5$\pm$0·3 atoms % excess.

Enzymic hydrolysis of yeast ^{15}N-labelled protein. Yeast protein was suspended in water (1 g./100 ml.) and brought to pH 1·5 with N-HCl (pH meter). The suspension was incubated at 37° under a layer of toluene after the addition of pepsin (The Armour Laboratories, Hampden Park, Eastbourne), 10 mg./g. of protein. The pH was adjusted from time to time and a further quantity of pepsin added after 3 days. The pH was then brought to 8·2 with N-NaOH after a further 8 days and pancreatin (Pancrex, high-potency pancreatin powder, Paines and Byrne Ltd., Greenford) was added (10 mg./g.). The pH was adjusted from time to time throughout a further 25 days, more pancreatin being added after 15 days. The yellow supernatant liquid was then separated from insoluble residue by centrifuging. The dry insoluble material contained 6 mg. of N/g. The extent of hydrolysis, 61%, was estimated by the ninhydrin method of Van Slyke, MacFadyen & Hamilton (1941) before and after complete hydrolysis with 3N-HCl for 16 hr. at 110°. Amide N (6·4% of total N) was estimated on a portion of the acid hydrolysate by the method outlined for urinary NH_3 below.

Recovery of ^{15}N. The ^{15}N residues from the media and from the washings after distillation of the solvents were evaporated with dil. H_2SO_4. Trichloroacetic acid was removed by repeatedly washing with ether. The ^{15}N was then converted into $(^{15}NH_4)_2SO_4$ by digestion with conc. H_2SO_4, the overall recovery being 85%.

Experimental subjects. These were normal healthy ambulant adults aged between 30 and 40 years. In the first two experiments (C1 and A1), subjects were on a known intake of N. Experiment J1 was carried out with the subject on a free diet. Yeast protein (about 0·8 mg. of

^{15}N/kg. body wt.) was taken just before breakfast in all initial experiments and urine collections were made at suitable intervals during the first 24 hr., continuing in 24 hr. lots over the subsequent 2 days. Specimens were stored at 4° under toluene. Analyses for NH_3, urea and total N were made in all specimens. Faeces were collected in 24 hr. lots for 6 days in two experiments and for 3 days in one, and stored at −30°. In subsequent experiments (C 2 and 3, A 2, 3 and 4), protein or protein hydrolysate (at the same ^{15}N dose as previously in three experiments; in two experiments at a lower dose) in 250 ml. of water was administered after taking 500 ml. of tap water, after a 12 hr. fast and with the subjects on free diets. Water (250 ml.) was given hourly to maintain a good urine flow and a normal meal was allowed 3 hr. after the commencement of each experiment. The second protein-hydrolysate run (A 3) was carried out under longer fasting conditions, the first meal being taken 10 hr. after the dose of ^{15}N was given. During these experiments urine collections were made at 30 min. or 20 min. intervals for the first 2 hr. and at suitable intervals up to 25 hr. Analyses for NH_3, urea, and α-amino N in the later experiments were made on the early specimens of urine. Subsequent urinary and faecal collections over 3 days were made as in the previous experiments. Venous blood was withdrawn at intervals during the experiments for determination and isolation of urea or α-amino N and collected over potassium oxalate or heparin respectively. All subjects were weighed daily at noon throughout each experimental period.

Fractionation of urinary nitrogen. Estimations of total N and free NH_3 were made in duplicate by methods which allowed for the simultaneous isolation of about 1 mg. quantities of N for ^{15}N excess determination. Hippuric acid was isolated for ^{15}N determination only.

Total nitrogen. Amounts of urine equivalent to about 5 mg. of N_2 were digested until clear (0·5–1 hr.) with 3 ml. of conc. H_2SO_4 and 3 g. of K_2SO_4–$HgSO_4$ mixture. After treatment with zinc dust to prevent interference by Hg^{2+} ions (King & Wootton, 1956), NH_3 was distilled into 2% boric acid and titrated with 0·1 N-H_2SO_4 with Sher's indicator (Sher, 1955).

Urinary ammonia. Estimation of this fraction was carried out by method A described by Berlin, Neuberger & Scott (1956). Ammonia was aspirated into 0·02 N-H_2SO_4 and back-titrated with NaOH and methyl red indicator screened with methylene blue. Preliminary experiments, with urine to which known amounts of NH_3 were added, showed recoveries of not less than 98·5%. Isolation of NH_3 by this method and by the phosphotungstate method from a sample of urine containing $^{15}NH_3$ (Berlin *et al.* 1956) gave closely similar values for ^{15}N excess (0·067 and 0·069).

Urinary urea. Urinary urea was estimated by treatment of samples of urine with urease (Hynson, Westcott and Dunning Inc., Baltimore, Md., U.S.A.) and aeration of the resultant total NH_3 into standard acid. Values for free urinary NH_3 were then subtracted. Urea N for ^{15}N determination was then isolated by method B, as outlined by Berlin *et al.* (1956).

α-Amino nitrogen. Samples of urine were concentrated *in vacuo* to about one-tenth of their volumes and then treated with 3 vol. of ethanol (Westall, 1952). After standing for 24 hr. at 0°, the solutions were filtered, the residue was washed with small quantities of cold 80% ethanol and the clear filtrate and washings were evaporated under reduced pressure until viscous, and finally diluted with water to about one-fifth of the original volume. Charcoal, washed with acetic acid (Partridge, 1949), was then added (about 1 g./30 ml. of urine) and the urine shaken for 30 min., giving a colourless solution after filtration. The urines were then run on to a column of Dowex 50 resin (3 cm. × 1·7 cm.), 200–400 mesh in the H^+ form, at about 50 ml./hr. The amino acids were displaced with aq. 0·05 M-NH_3 soln. after washing the column with water until the pH was greater than 5·0, and were collected in 25 ml. fractions. Ninhydrin-reacting fractions were pooled and evaporated under reduced pressure almost to dryness and transferred to stoppered tubes. Ammonia was removed from the amino acids by ninhydrin as described by Sobel, Hirschman & Besman (1945), and aspirated into 2% boric acid.

Isolation of hippuric acid. The charcoal residues from the urinary α-amino N isolations were eluted on a filter with 10% (w/v) phenol (Dalgliesh, 1955) until the filtrate became colourless. After repeated evaporation *in vacuo* to remove the phenol, the filtrates were combined with the first washings from the Dowex columns and the combined solutions brought to pH 1·0 with HCl and repeatedly extracted with ethyl acetate. Hippuric acid which did not depress the melting point of an authentic specimen was recovered in thick needles on evaporation of the ethyl acetate and twice recrystallizing the sticky residue from water. Chromatography in butanol–acetic acid–water (12:3:5, v/v) on a Whatman no. 1 paper after hydrolysis of a small quantity of the hippuric acid with 3 N-HCl yielded a single ninhydrin-reacting spot with R_F identical with that of glycine.

Faecal nitrogen. A 24 hr. sample of faeces was emulsified in a blender with 400 ml. of water, and total N was determined on a measured specimen equivalent to about 2–3 g. of faeces. The digestion and estimations were carried out as for total urinary N except that 5 ml. of H_2SO_4 was used.

Trichloroacetic acid-soluble faecal nitrogen. Emulsified faeces (2–3 g.) were extracted three times with 25 ml. portions of 5% trichloroacetic acid. The combined extracts were made up to 100 ml. with water and suitable samples taken for determinations of total N.

Blood urea. This was estimated by the method of Van Slyke & Cullen (1914).

Plasma amino nitrogen. Heparinized plasma was deproteinized with an equal volume of 5% trichloroacetic acid and after 5 min. was centrifuged at 3000 rev./min. The residue was extracted twice with 5% trichloroacetic acid in amounts equivalent to 0·25 vol. of plasma, followed by removal of the acid by repeatedly washing the combined extracts with ether until the pH was greater than 5·0. Excess of ether was removed by bubbling air through the aqueous solution. Incubation of the deproteinized plasma with urease at 37°, followed by the adsorption of the resultant NH_3 on Decalso F, removed the urea. Ammonia was then released from the amino acids as described above. Test experiments with a solution of amino acids equivalent in composition and concentration to human post-absorptive plasma (Stein & Moore, 1954) showed that recoveries of NH_3 from the amino acids by the method of Sobel *et al.* (1945) were similar before and after treatment with 5% trichloroacetic acid. Since higher values were obtained when urea at normal concentrations was added to the

solution of amino acids, this was removed by urease and adsorption on to Decalso F. Finally, no effect could be detected when physiological amounts of uric acid and creatinine were added.

Excess of ^{15}N *determinations.* All specimens of NH_3 for determination in the mass spectrometer (about 1 mg. of N) were further digested for 16 hr. with H_2SO_4 and the K_2SO_4–$HgSO_4$ catalyst after distillation to remove traces of indicator. Excess of ^{15}N was determined by the hypobromite method outlined by Sprinson & Rittenberg (1949*a*).

Enzymic hydrolysis of yeast proteins with large amounts of trypsin and chymotrypsin. In these experiments crude yeast protein containing about 9·6% of N was used; this material was contaminated with some insoluble polysaccharide. The enzymic digestion was carried out with shaking at 37° in a solution of KH_2PO_4–Na_2HPO_4 (0·1 M; pH 7·2, measured electrometrically) containing $CaCl_2$ (2 mM). Slight cloudiness developed in the solutions owing to precipitation of some of the calcium as calcium phosphate. The enzymes used were crystalline trypsin and chymotrypsin. At suitable intervals of time 2 ml. of the suspension was withdrawn, mixed with 2 ml. of 20% (w/v) trichloroacetic acid and the suspension was made up to 5 ml. After standing for 2 hr. the mixtures were filtered and two 2 ml. samples were used for estimation of N. This was done by heating the samples under reflux for 18 hr. after addition of 1·5 ml. of conc. H_2SO_4 containing SeO_2, $CuSO_4$ and K_2SO_4. Controls were done by incubating the yeast protein by itself and the two enzymes without yeast protein.

RESULTS

Excretion of labelled nitrogen in the urine and faeces

In three experiments yeast protein (approx. 0·8 mg. of ^{15}N/kg. body wt.) was given to three normal subjects in the morning before breakfast. In two experiments, A 1 and C 1, the subjects were on diets providing 99 and 80 g. of protein/day respectively, but the caloric intake was insufficient with the result that N balance was negative. In the third experiment, J 1, a free diet was allowed and N balance was not determined, but there was no loss of weight. Full details of one experiment (A 1) are given in Table 1, and the course of excretion of labelled N in the urine in the other two experiments (C 1 and J 1) is shown in Fig. 1. The total labelled N excreted in the urine in the first 72 hr. was 30·5%

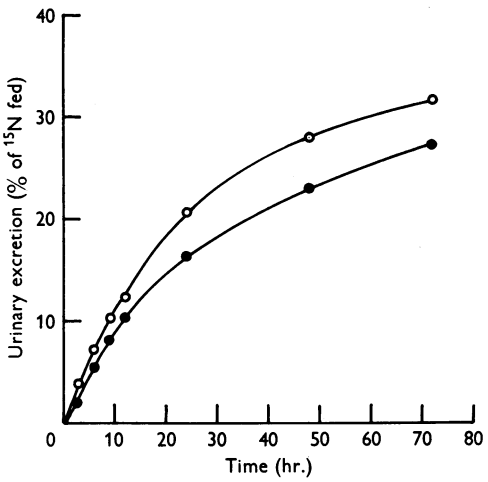

Fig. 1. Cumulative excretion of ^{15}N in the urine. Results are given as a percentage of the ^{15}N supplied as yeast protein. Other experimental details are given in the text. ○, Expt. C 1; ●, Expt. J 1.

Table 1. *Excretion of labelled nitrogen in the urine of a normal subject who had been fed yeast* ^{15}N*-labelled protein (Expt. A 1)*

The normal subject weighed 83·5 kg. (fully clothed) and received 75·25 mg. of ^{15}N in the form of yeast protein after an overnight fast. Immediately after the protein meal, the subject was given breakfast. For 2 days before the labelled protein was given and for 4 days afterwards the diet supplied 2320 kcal./day and 15·8 g. of N/day. Owing to the low caloric intake the daily N balance was negative, varying during the period stated between −1·5 g. and −4·2 g./day.

	Total nitrogen			Ammonia			Urea		
Time (hr.)	N (mg.) *a*	^{15}N content (atom % excess) *b*	Cumulative % excretion of ^{15}N fed $\frac{c}{75{\cdot}25}$ where $c = \Sigma ab$	N (mg.) *d*	^{15}N content (atom % excess) *e*	Cumulative % excretion of ^{15}N fed $\frac{f}{75{\cdot}25}$ where $f = \Sigma de$	N (mg.) *g*	^{15}N content (atom % excess) *h*	Cumulative % excretion of ^{15}N fed $\frac{i}{75{\cdot}25}$ where $i = \Sigma gh$
0–1·5	1 242	0·070	1·16	25·9	0·301	0·10	1 059	0·058	0·82
1·5–3	1 438	0·108	3·22	39·6	0·207	0·21	1 303	0·105	2·63
3–6	2 543	0·098	6·54	68·0	0·094	0·30	2 182	0·107	5·75
6–9	2 360	0·088	9·30	84·0	0·058	0·36	1 971	0·087	8·01
9–12	1 700	0·082	11·15	77·0	0·054	0·42	1 358	0·092	9·68
12–24	7 370	0·062	17·20	291·0	0·028	0·53	5 939	0·070	15·20
24–48	17 300	0·036	25·45	635	0·028	0·76	14 750	0·040	23·00
48–72	18 750	0·020	30·47	740	0·015	0·91	15 460	0·021	27·30
120–144	15 000	0·010	2·00*						
288–312	13 300	0·004	0·71*						

* Non-cumulative values.

of the amount fed in Expt. A 1, and the corresponding figures for the other two experiments were 31·9 and 27·4 %. In two further experiments in which yeast ^{15}N-labelled protein was fed and the subjects were allowed free diets (A 2 and C 2), 26·3 and 26·6 % of the administered ^{15}N was excreted in the urine in the first 72 hr. The mean value for the 72 hr. urinary excretion was 28·5 % when whole protein was used, and individual variations in the five experiments did not exceed 12 % of this figure. Urinary excretion for 72 hr. when yeast ^{15}N-labelled protein hydrolysate was substituted (A 3 and C 3) gave values of 23·5 and 28·1 %, the former low excretion possibly being due to the withdrawal of some 250 ml. of blood during the first 12 hr. of the experiment. The figures for the proportion of the fed ^{15}N excreted in the urine for the first 24 hr. were more variable, and in the five protein experiments were 20·7, 16·4, 17·2, 16·1 and 15·1 % (mean value 17·1 %) and for the protein hydrolysate 18·8 and 13 %. The proportion of the fed ^{15}N which appears in the urine in the first 24 or 72 hr. is thus appreciably smaller when either whole-yeast protein or protein hydrolysate is given, than if a single amino acid such as glycine is supplied. Sprinson & Rittenberg (1949*b*) found that if [^{15}N]glycine is given in amounts similar to those of the yeast protein used in the present experiments to a person on a normal diet, supplying approx. 0·2 g. of N/kg. body wt./day, 30 % of the ^{15}N is excreted in the urine in the first 24 hr. and 45 % in the first 72 hr.; similar results were obtained by ourselves. The subjects given yeast protein had a daily intake of 0·16–0·19 g. of N/kg. body wt. These findings for yeast protein are in agreement with the results of White & Parson (1950), who suggested that this difference might be caused by the slow absorption of the protein, which has first to be hydrolysed. However, data presented later in this paper indicate that enzymic hydrolysis of the protein is not rate-limiting to any appreciable extent and the fast elimination of the labelled N, when glycine is fed, is likely to be caused by an imbalance of the type discussed by Geiger (1947) or by differences in the rates by which the N of various amino acids is converted into urea. That such differences may be fairly large is indicated by the data of Wu & Sendroy (1959).

The proportion of the fed ^{15}N eliminated in the faeces varied markedly in different experiments. In the experiment described in Table 1 the percentage of the fed ^{15}N excreted in the faeces during the first 72 hr. was 5·35 % of the amount fed, and in other experiments the corresponding values were 3·62 and 6·25 %. When yeast-protein hydrolysate was given, figures of 4·7 and 6·0 % were obtained. In the few experiments when collection of the faeces was continued beyond 3 days there was generally little more ^{15}N excreted. Thus in one experiment 0·85 % of the labelled dose was obtained in the second 3-day faeces collection, whereas only an insignificant amount was found in the faeces on the thirteenth day of the experiment. A similar situation applies to the total urinary N (Table 1).

However, in one subject only 0·18 % of the fed ^{15}N was excreted in the faeces during the first 3 days, whereas 3·30 % was obtained in the second 3-day period, the amount having already decreased sharply from 0·064 atom % excess on the fifth day to 0·018 on the sixth day of the experiment. Sharp *et al.* (1956) in similar experiments with ^{15}N-labelled yeast found a somewhat higher amount, 10 %, of ^{15}N eliminated in the faeces over 6 days, but these authors fed intact dried-yeast cells.

About 25–30 % of the faecal N was soluble in trichloroacetic acid; the isotope content of the trichloroacetic acid-soluble N was generally closely similar to that of the insoluble faecal N.

Contents of ^{15}N in blood urea and urinary urea

The urinary urea was found to be already significantly labelled in the specimen collected in the first 30 min. after ingestion of the labelled protein (Table 4); indeed it was found in several experiments that the ^{15}N content of the urinary urea obtained during the first 20 or 30 min. of the experiment varied between 15 and 22 % of the maximum value. The latter was reached within about 60 min. (Table 4, Fig. 2) and then remained constant for about 4 hr., to decrease very sharply after that time.

The blood urea (Table 2) showed a similar behaviour; values at 20 min. were already 40 % of those obtained at the maximum which was reached at about 1 hr. and which was maintained for a further approx. 4 hr. In several experiments (e.g. Table 4) in which blood urea and urinary urea were examined simultaneously a plateau was observed which extended for about 4 hr. In these experiments the isotope contents were constant generally within ± 3 %, but the blood-urea values were invariably lower than the urinary-urea figures, the differences being about 10 %. A similar finding in one experiment was reported by San Pietro & Rittenberg (1953*a*), who injected [^{15}N]urea intravenously. It appears that the assumption made by these authors that the mixing of the injected labelled urea with the preformed urea of the body is complete within about 40 min. is not strictly correct. It seems more likely that a steady state is reached and that differences in isotope content between urea present in different 'compartments' of the body may persist beyond 40 min. An alternative explanation for the differences in the isotope content between blood and urinary urea

Table 2. *Comparison of the labelling in the blood urea after ingestion of yeast* ^{15}N*-labelled protein (Expt. C 2) and of yeast* ^{15}N*-labelled protein hydrolysate (Expt. C 3)*

The subject (body wt. 82 kg.) consumed 0·89 mg. of ^{15}N/kg. in Expt. C 2 and 0·83 mg. of ^{15}N/kg. in Expt. C 3. The subject fasted for 12 hr. before ingestion and for 3 hr. after ingestion. —, Not determined.

	Expt. C 2		Expt. C 3	
Time (hr.)	Urea N (mg./100 ml.)	^{15}N content (atom % excess)	Urea N (mg./100 ml.)	^{15}N content (atom % excess)
0·33	10·4	0·049	14·2	0·079
0·67	10·2	0·099	14·1	0·108
1·0	12·2	0·121	14·0	0·114
1·5	12·2	0·127	13·3	0·118
2·0	10·0	0·127	13·0	0·117
3·0	10·3	—	12·9	0·120
4·5	10·8	0·127	13·7	0·118
6·0	11·7	0·117	13·9	0·111
8·0	—	—	13·7	0·101
24·0	14·1	0·059	—	—

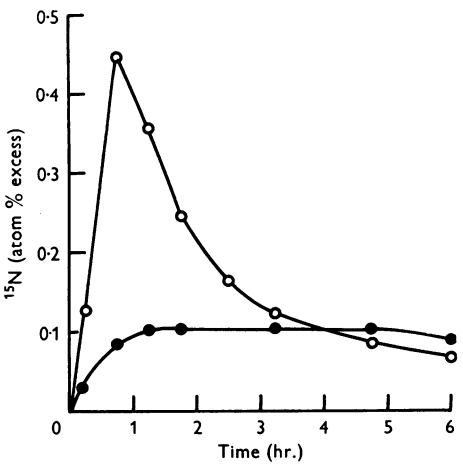

Fig. 2. Change of ^{15}N content with time of urinary ammonia and urinary urea. Expt. A 2: subject (83·5 kg. body wt.) received 0·9 mg. of ^{15}N/kg. body wt. of whole-yeast ^{15}N-labelled protein. The average ^{15}N content for the time interval is plotted at the mid-point of the interval. ○, Urinary ammonia; ●, urinary urea.

may be that there is discrimination between [^{14}N]urea and [^{15}N]urea during glomerular filtration or more likely during tubular reabsorption.

The differences in the urea values between experiments in which labelled protein or labelled hydrolysate were fed are generally very small. Thus the ratios in the maximum isotope contents of blood urea found in Expts. C 2 and C 3 respectively were exactly proportional to the amounts of ^{15}N fed (Table 2). However, the values obtained at 20 min. were lower in the protein experiment than in the hydrolysate experiment, being 65 % of the maximum figure in the latter as compared with 40 % in the former. This suggests that enzymic hydrolysis of the protein may delay absorption by at most 10 min.

Content of ^{15}N *in urinary ammonia*

The maximum ^{15}N contents of the urinary NH_3 were 3·5–5·0 times those of the urinary urea both in the protein (e.g. Fig. 2 and Table 3) and in the hydrolysate experiments. The ^{15}N content of the NH_3 rose and fell much more sharply than the corresponding urea figure; thus at about 4·0–4·5 hr. the ratio of atom % excess of ^{15}N in the NH_3 to that of urinary urea was unity (Fig. 2) and then fell to values well below 1·0.

Again the NH_3 collected during the first 30 min. in the hydrolysate experiments appeared to be somewhat more highly labelled than in the protein experiments (see Table 3 and Fig. 3), but the large changes in the ^{15}N content of the NH_3 occurring over a short period of time render a comparison between different experiments in which urine is collected over 30 min. somewhat difficult.

The third urine specimen (60–90 min.) always had a lower ^{15}N content than the second specimen (30–60 min.), the difference varying between 10 and 20 %. It would seem likely therefore that the maximum labelling in the urinary NH_3 occurred about 50 min. after ingestion of the labelled protein.

Content of ^{15}N *in plasma and urinary amino acids*

Only a relatively small number of values have so far been obtained for the changes in ^{15}N content of plasma and urinary amino acids (Table 5). However, it appears that the maximum ^{15}N content in the plasma amino acids occurs some time between 35 and 50 min. after ingestion of the labelled protein. The same appeared to apply to an experiment in which a hydrolysate was fed. The values decrease sharply and at 195 min. the labelling in the plasma amino acids has fallen to 27 % of the

maximum figure. So far as it is possible to compare the ^{15}N contents of the mixed amino acids of urine and of plasma it would appear that the former are higher than the latter. This situation is similar to that found for urea (Table 4). In both cases the urinary material is derived from the arterial blood, whereas the plasma was obtained from a vein. It is hoped to examine this point further by a comparison of arterial and venous samples obtained at the same time. But it is important to remember that the amino acids present in blood are a heterogeneous mixture containing not only amino acids derived directly from dietary or tissue proteins, but also compounds such as taurine or methylhistidine which are products of secondary reactions (see Stein & Moore, 1954). This also may explain why labelling in the urinary NH_3 was higher than that found in either the plasma or urinary amino acids. Moreover, a comparison between the labelling of the plasma amino acids and of the urinary amino acids is made difficult by the fact that the relative composition of these two fractions is by no means identical owing to differences in the rates by which various amino acids present in the glomerular filtrate are reabsorbed by the tubules.

Table 3. *Comparison of the labelling in the urinary ammonia of a subject after ingestion of yeast* ^{15}N*-labelled protein (Expt. C 2) and of yeast* ^{15}N*-labelled protein hydrolysate (Expt. C 3)*

For experimental details see Table 2. —, Not determined.

Time (hr.)	Isotope content (atom % excess) Protein	Hydrolysate
0–0·5	0·121	0·241
0·5–1·0	0·490	0·623
1·0–1·5	0·439	0·435
1·5–2·0	0·304	0·292
2·0–3·0	0·205	0·207
3·0–3·5	—	0·172
3·0–4·5	0·131	—
4·5–5·0	—	0·111
4·5–6·0	0·101	—
6·0–6·5	—	0·088
10·6–11·1	—	0·055
24·2–24·7	—	0·038

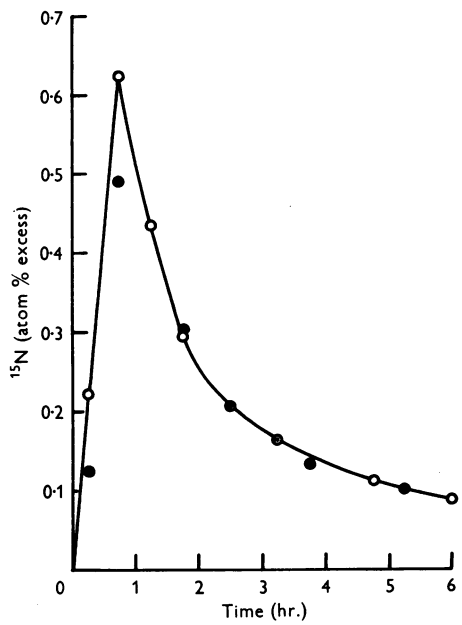

Fig. 3. Comparison of changes of ^{15}N content with time of urinary ammonia after ingestion of whole yeast ^{15}N-labelled protein and yeast ^{15}N-labelled protein hydrolysate. ●, Expt. C 2 after feeding whole-yeast protein; ○, Expt. C 3 after feeding protein hydrolysate. Plotting is as for Fig. 2. Further details are given in Table 2 and the text.

Further information is obtained by the results shown in Table 6. In this investigation urines from Expts. A 2 (Table 4) and C 2 (Table 2) were pooled and the ^{15}N contents of amino acid N and of hippuric acid measured. The hippuric acid was material present in the urines without previous administration of benzoate. In both Expts. C 2 and A 2 yeast protein and not a hydrolysate had been fed. In these pooled urines the labelling of the amino acid N was (apart from the first hour sample) considerably higher than in the NH_3 N and the maximum value was obtained in the second-hour urine specimen. The hippuric acid was even more highly labelled than were the free amino acids. When yeast-protein hydrolysate was given, the ^{15}N content of the urinary amino acids in the specimens obtained after the first hour was 2–3 times that of the urinary NH_3 and at most twice

Table 4. *Comparison of the labelling in urea of venous blood and in urea of urine after ingestion of* ^{15}N*-labelled proteins (Expt. A 2)*

The subject (body wt. 83 kg.) consumed 0·9 mg. of ^{15}N/kg. body wt. in the form of yeast protein. He fasted for 12 hr. before ingestion and for 3 hr. afterwards. —, Not determined.

Time (hr.)	Blood urea. Isotope content (atom % excess)	Urinary urea Time (hr.)	Isotope content (atom % excess)
0·35	0·040	0–0·5	0·016
0·67	0·068	0·5–1·0	0·081
1·02	0·081	1·0–1·5	0·101
1·52	0·091	1·5–2·0	0·101
2·04	0·087	—	—
3·02	0·098	3·0–3·5	0·101
4·53	0·087	4·5–5·0	0·101
6·03	0·085	6·0–6·5	0·085
8·00	0·075	25·5–26·5	0·049

Table 5. *Comparison of the labelling in amino acids in venous blood and those in urine with that of urinary ammonia*

The subject (body wt. 83 kg.) received yeast ^{15}N-labelled protein hydrolysate (Expt. A3) at a dose of 0·60 mg. of ^{15}N/kg. and whole-yeast labelled protein (Expt. A4) at a dose of 0·44 mg./kg. The figures in parentheses after the ^{15}N contents of the plasma amino acids indicate the times (in hr.) at which the blood samples were taken.

	Urine			Plasma.		
	Ammonia N	Amino acid N		Amino acid N		
Time	(atom % excess)			(atom % excess)		
(hr.)	*a*	*b*	*a/b*	*c*	*a/c*	*b/c*
Expt. A3						
0–0·5	0·102	0·142	0·72	0·089 (0·25)	1·15	1·60
0·5–1·0	0·322	0·357	0·91	0·227 (0·75)	1·42	1·57
1·0–1·5	0·312	0·674	0·46	0·209 (1·25)	1·40	3·20
1·5–2·0	0·208	0·522	0·40	0·151 (1·75)	1·38	3·50
3·0–3·5	0·111	0·371	0·30	—	—	—
4·5–5·0	0·085	0·266	0·32	—	—	—
6·0–6·5	0·068	0·235	0·29	—	—	—
8·5–9·5	0·055	0·206	0·27	—	—	—
Expt. A4						
0–0·33	0·040	0·028	1·41	0·018 (0·17)	2·2	1·56
0·33–0·67	0·158	—	—	0·139 (0·5)	1·14	—
0·67–1·0	0·269	—	—	0·137 (0·83)	1·98	—
1·0–1·5	0·203	0·157	1·29	0·114 (1·25)	1·79	1·39
1·5–2·0	0·145	0·124	1·17	—	—	—
3·0–3·5	0·050	0·050	1·01	0·038 (3·25)	1·32	1·31

Table 6. *Comparison of ^{15}N contents of urinary ammonia, mixed urinary amino acids and hippuric acid after feeding of labelled protein*

Appropriate specimens obtained in Expts. A2 and C2 (Tables 3 and 4) were pooled and the ^{15}N contents of the amino acid fraction and of crystalline hippuric acid were determined as described. The ammonia values were calculated from the ammonia contents and the isotope analyses of the individual specimens.

Time (hr.)	NH_3	Amino acid N	Hippuric acid N
	Atom % excess of ^{15}N		
0–1	0·310	0·312	0·487
1–2	0·334	0·530	0·874
2–3	0·129	0·350	0·417
3–4·5	0·112	0·185	0·168

that of the plasma amino acids (Table 5). The ^{15}N content of the various amino acids present in the urine may change at greatly different rates with time in different experiments, owing to factors not clearly understood, and a really satisfactory interpretation will only be possible when at least some of the amino acids in the urine are isolated and the changes of ^{15}N content with time are measured in pure compounds.

It is difficult to draw general conclusions from our fairly limited data, but it is likely that the plasma amino acid N figures present a more consistent picture than the corresponding urinary values. This is suggested by a comparison of the plasma results presented in Table 5 (Expts. A3 and A4) and some additional data as yet unpublished. In the experiments described in Table 5 the time curves of the ^{15}N content of the plasma amino acids are fairly similar, if the differences in the amount of ^{15}N supplied are taken into account. But it must be remembered that in this case, too, the ^{15}N contents of the amino acids derived from the fed labelled protein must be considerably diluted by compounds such as taurine or the methylhistidines, which presumably have low ^{15}N contents, within the first 1–2 hr. after ingestion of the labelled protein.

Enzymic hydrolysis of yeast protein

Enzymic experiments *in vitro* were prompted by the finding that the rates of absorption of labelled yeast protein and of labelled hydrolysate were closely similar. Borgström, Dahlqvist, Lundh & Sjövall (1957) investigated the concentrations of trypsin and chymotrypsin in the intestinal contents of human subjects and found that these varied in the duodenum and jejunum between 0 and 350 μg./ml. for each of the two enzymes. When trypsin concentration was related to the time of ingestion of a test meal, variations were smaller. The average concentration of trypsin was about 300 μg./ml. and this value was reached 10–20 min. after ingestion of a meal. In the first experiment the concentrations of both enzymes were 2000 μg./ml., i.e. more than twice the highest value which has been observed in normal human subjects. Under these conditions about half the protein N

was changed in 5 min. into a form which is non-precipitable by trichloroacetic acid. In the second experiment the concentrations of trypsin and chymotrypsin were reduced to 400 μg./ml. but the rate of digestion was not greatly reduced. Thus about 40% of the protein was broken down in 5 min. and approx. 60% in 10 min. (Fig. 4).

In these experiments trichloroacetic acid was used to separate proteins and trichloroacetic acid-insoluble peptides from trichloroacetic acid-soluble peptides. It is thus likely that the values given in Fig. 4 are misleading if the action of trypsin and chymotrypsin is considered in terms of the proportion of susceptible peptide bonds split. However, the main aim of these experiments was to investigate what percentage of the protein was degraded in a given time and for this purpose trichloroacetic acid appeared most suitable and convenient. With the very high concentrations of enzymes used, autodigestion had to be considered and it was found to be large, but the values given in Fig. 4 have been corrected accordingly. This was done by incubating the two enzymes alone and deducting the values of trichloroacetic acid-soluble N from the corresponding values in the main experiment.

The pH chosen in these enzymic digestions was lower than 7·8, which is generally considered the optimum for most peptide substrates. However, Borgström *et al.* (1957) have shown that the pH in the duodenum is generally about 6·0 and rises in the lower ileum to about 7·8. A value of 7·2 appeared to be close to the average pH of the natural environment of these enzymes.

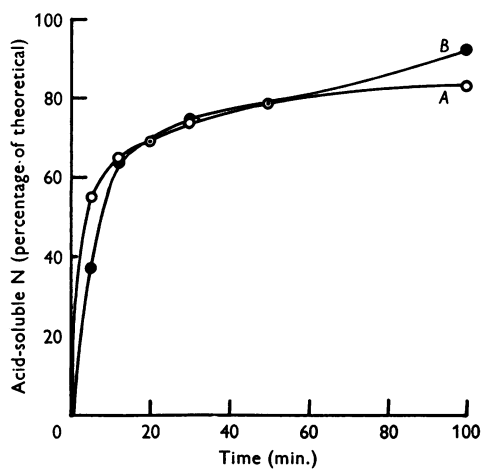

Fig. 4. Rate of hydrolysis *in vitro* of yeast protein by trypsin and chymotrypsin. Expt. *A*, concentrations of 40 mg. of both enzymes were used in a volume of 20 ml. containing phosphate buffer, pH 7·2, and 250 mg. of yeast protein. Expt. *B*, composition was the same except that amounts of the enzymes were both 8 mg. Temperature, 37°. Rate of hydrolysis was estimated by measuring the amount of N soluble in 20% trichloroacetic acid. Corrections for autodigestion of the enzyme were made as described in the text.

DISCUSSION

Comparison of the rate of absorption of whole protein with that of protein hydrolysate

The results of the present investigation indicate that the rate of absorption in experiments in which whole-yeast protein is fed is more or less the same as in experiments in which a hydrolysate is used. There are slight differences which are probably significant. Thus the blood urea was labelled more highly 20 and 40 min. after ingestion when ^{15}N-labelled hydrolysate was given than when ^{15}N-labelled whole protein was fed (Table 2). Similarly, urinary ammonia was labelled more highly during the first hour after hydrolysate feeding than after protein feeding (Table 3) and this seems to apply also to the ^{15}N content in the plasma amino acids (Table 5). But such differences, if they are significant, are relatively slight. During the first hour after ingestion the ^{15}N content in the various plasma and urine fractions examined rises steeply and the differences observed could be explained by assuming that the average delay in overall absorption caused by enzymic hydrolysis of the proteins to peptides or amino acids does not take more than 10 min.

The idea that there is no marked difference between the rate of absorption after feeding whole protein and after feeding a hydrolysate is not completely novel. Thus Janney (1915) measured the time course of the excretion of extra glucose and extra nitrogen after administration in three different experiments of glucose, protein and acid protein hydrolysate to phlorrhizinized dogs. He found that there was very little difference between protein and hydrolysate. More recently, Gupta, Dakroury & Harper (1958) examined intestinal contents of rats which had been given test meals containing about 75% of sucrose, 5% of corn oil and 15% of whole protein or amino acid mixtures. These authors noted that there was no significant difference in the rate of disappearance of the fed nitrogen from the gut between the experiments in which protein and the experiments in which amino acids were fed. The question arises whether this fast rate of overall absorption of whole protein indicated in the present experiments and suggested by the earlier work can be reconciled with the assumption tacitly made or expressly stated by most workers in this field that protein is first broken down to amino acids by the known proteolytic enzymes pepsin, trypsin and chymotrypsin before absorption by the mucosa can occur.

Fisher (1954) discussed this problem in some detail and came to the conclusion that the evidence for the belief that amino acids only are the end products of digestion of protein is unsatisfactory and is at variance with some experimental facts. The various possibilities to explain these facts may now be considered.

Protein may be absorbed as such by the mucosal cells, and is broken down there or is transported by the portal route or is removed by the lymphatics to the liver or systemic circulation. Absorption of whole protein by the mucosa of the normal adult is unlikely to occur, as opposed to the newborn of many species in whom large amounts of colostrum proteins are absorbed in the first 48 hr. of life. But even here there is considerable selectivity; thus Bangham & Terry (1957) have shown that in rats 100 times as much globulin is absorbed as albumin and a similar situation exists in the pig (McCance & Widdowson, 1959); in the young calf no such discrimination is observed (Bangham, Ingram, Roy, Shillam & Terry, 1958). However, it is generally agreed that in all species examined substantial absorption of whole protein ceases after the first 2 weeks of life, the one possible exception being homologous native plasma protein (Dent & Schilling, 1949). As pointed out by Newey & Smyth (1959), immunological evidence indicating that intestinal absorption of whole protein can occur is not relevant to the problem.

We must therefore assume that some hydrolysis of protein occurs before absorption. The work of Borgström *et al.* (1957) shows that duodenal contents of man may contain about 200–800 μg. of trypsin and of chymotrypsin/ml. of fluid within a short time after a stimulus is applied. It was found in the present work that with concentrations of these two enzymes of 400 μg./ml. well over 50% of the protein is converted into peptides soluble in trichloroacetic acid in 10 min. It seems therefore that the conversion of protein into small peptides is likely to be a fast process *in vivo*. The percentage of susceptible peptide bonds split may not be so large as our results suggest and it seems likely that conversion of such peptides into amino acids is normally largely carried out by enzymes present in the intestinal mucosa. Indeed, Johnston & Wiggans (1958) and Newey & Smyth (1959) have shown that dipeptides can pass across the mucosal barrier and it is probable that this will apply also to polypeptides. Evidence was obtained by Newey & Smyth (1959) indicating that the peptides are hydrolysed inside the mucosal cells, and it is thus reasonable to assume that digestion of proteins occurs by co-operation of pepsin, trypsin and chymotrypsin and by 'erepsin', which is a mixture of peptidases present in mucosal cells of the gut (Wright, Jennings, Florey & Lium, 1940).

The overall process of digestion of protein as measured in our experiments is remarkably fast. Maximum ^{15}N content in the amino acids of systemic blood is reached between 30 and 50 min. (Table 5) and may occur somewhat earlier in the portal blood. Urinary ammonia also appears to have its highest ^{15}N content before 1 hr. is passed (Tables 3 and 5; Figs. 2 and 3). At 2 hr. these figures have decreased to about 50% of their maximum value. It is difficult to arrive at any quantitative data on the rate of absorption from these figures for the following reason. The appearance of the label in certain fractions of systemic blood or in urine is the result of a series of reactions after the actual absorption. The amino acids are presumably first transported by the portal vein to the liver, where they mix with fairly large amounts of unlabelled amino acids. From there they are transferred to the systemic blood where further mixing occurs, both with the unlabelled amino acids present in the blood and with amino acids found in tissues other than the liver. This interchange of labelled amino acids between blood and tissue and the reversible incorporation of amino acids into proteins and other compounds of short turnover time will go on in both directions for a long time after absorption is completed, and labelled amino acids will thus circulate in the blood at a time when no appreciable amount of ^{15}N is transferred across the mucosa.

But the data presented allow the conclusion that a significant amount of absorption already occurs within 15 or 20 min. after ingestion (Tables 2, 4 and 5), that the rate of absorption is at its maximum at about 45 min., but possibly earlier, and that absorption may be largely completed within 75 or 90 min.

The question arises why absorption of protein under our experimental conditions is so much faster than was observed by other workers. The reasons, we believe, are that in the experiments under consideration the amount of protein is small (< 3 g.) and that the protein was not fed together with either carbohydrate or, particularly, fat, thus reducing gastric emptying time to a very low value. The fact that gastric emptying time is the rate-determining step in the overall absorption of test meals containing fat and carbohydrate in addition to protein is shown by the work of Gupta *et al.* (1958) and Rosenthal & Nasset (1958), who worked with rats. Hence our results cannot be applied quantitatively to protein absorption under normal conditions under which larger amounts of protein are consumed together with fat and carbohydrate.

Formation of urea and ammonia

The comments made above about the labelling of plasma amino acids as indicators of absorption of protein apply even more forcibly to urea. The

nitrogen atoms of this compound are supplied from the amino acids present in the liver and the urea formed will mix with the urea already present in the plasma, with the urea in the extracellular water and with the urea found in cells. San Pietro & Rittenberg (1953*a*) have calculated that urea is distributed over a volume of water equivalent to about 50% of body wt.; this would mean that in our experimental subjects total urea contents would be of the order of 5–7 g. of urea nitrogen. These authors also found that mixing of intravenously injected urea was essentially complete within 20–30 min. and that an amount of urea nitrogen equivalent to half the size of the urea pool is excreted every 9 hr. Thus in our experiments the rate of change of ^{15}N in the blood urea will be a function of the rate of absorption of labelled amino acids from the gut, of the rates of the mixing and of metabolic reactions of the amino acids as discussed above, of the rate of formation of urea in the liver, the rate of mixing of the newly formed urea with the large urea pool of the body and finally the rate of elimination of urea by the kidney. After 1·5 or 2 hr. the absorption of labelled nitrogen, if it occurs at all, will have only a negligible effect on the ^{15}N content of the blood urea or urinary urea. The plateau observed in the ^{15}N content of the blood urea between 1·5 and 4·5 hr. (Tables 2 and 4) indicates that the rate of addition of [^{15}N]urea by new formation from labelled amino acids is more or less equal to the rate of disappearance of labelled urea from the body by elimination through the kidney. This conclusion may seem somewhat surprising in view of the fact that the ^{15}N content of the plasma amino acids decreases sharply between 75 and 200 min. (Table 5; see also Table 6). However, the turnover of the urea pool is slow, relative to the rates of other reactions under consideration, and even in the absence of new formation of labelled urea the decrease in the ^{15}N content of blood urea between 2·0 and 4·5 hr. would not be expected to be more than 15%.

The high ^{15}N content of urinary ammonia (Tables 3, 5 and 6; Figs. 2 and 3) at about 30–90 min. after ingestion calls for a brief comment. It was first shown by Wu (1951) that during the first few hours after ingestion of L-[^{15}N]aspartic acid the ^{15}N content of the urinary ammonia is much higher than that of urea and similar results were later reported by Wu & Sendroy (1959) for several other L-amino acids. Van Slyke *et al.* (1943) have shown that in the dog at least 60% of urinary ammonia is derived from the amide group of glutamine. Whether this conclusion also applies to man is unknown and it is also uncertain whether the high ^{15}N content of the urinary ammonia observed after the injection of ^{15}N-labelled amino acids other than glutamine is caused by oxidative deamination of these amino acids or is due to a preliminary conversion of α-amino groups into the amide group of glutamine. In any case this question is largely irrelevant to the present discussion, and further experiments are required to settle this point. Both the maximum amounts of ^{15}N in urinary ammonia obtained in our experiments and the changes of ^{15}N content with time are of a similar order to those observed by Wu (1951) and by Wu & Sendroy (1959). However, we may provisionally use the ^{15}N changes in the urinary ammonia as a general indicator of protein absorption and assume that the ^{15}N in this fraction originates to some extent from all α-amino acids. The fact that the body 'pool' of ammonia is almost zero, the consequent sharp rise and fall in ^{15}N content, and the easy measurement make it a convenient tool for following protein absorption. However, it must be remembered that the isotope contents of the mixed amino acids and of hippuric acid (Tables 5 and 6) may be appreciably higher than that of ammonia.

SUMMARY

1. Yeast ^{15}N-labelled protein and yeast ^{15}N-labelled protein hydrolysate have been fed in amounts equivalent to 0·9–0·4 mg. of ^{15}N/kg. body wt. to healthy human subjects.

2. The proportions of fed ^{15}N in the urine at the end of 72 hr. after feeding either whole protein or protein hydrolysate were similar, and less than when a single ^{15}N-labelled amino acid was fed. Values at the end of 24 hr. periods, and for faecal elimination of the isotope, were more variable.

3. Comparison between the ^{15}N content of urinary urea and blood urea in the initial stages of the experiments showed consistently higher values of the former, and both showed almost maximum labelling within 1 hr. of ingestion of whole protein and protein hydrolysate.

4. The ^{15}N contents of urinary ammonia in both types of experiment rose sharply to maximum values in the collection period 30–60 min. after ingestion and fell rapidly thereafter. Plasma amino nitrogen showed similar time relationships; urinary amino nitrogen values were less consistent. It is considered that under the conditions of the experiment hydrolysis of protein within the gut is not a major rate-determining factor in its absorption.

5. In experiments at pH 7·2 and 37° *in vitro* with high concentrations of trypsin and chymotrypsin there was rapid breakdown of yeast protein to trichloroacetic acid-soluble material.

6. The significance of these findings is discussed in relation to the problems of digestion and absorption of protein in the human subject.

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Normal and Abnormal Di-iodo-L-Tyrosine Metabolism in Human Subjects

By CHRISTINA CAMERON

Medical Research Council, Department of Clinical Research, University College Hospital Medical School, London, W.C. 1

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Three human subjects who had been treated for myxoedema rapidly deiodinated intravenously administered [3:5-$^{131}I_2$]di-iodotyrosine, [^{131}I]iodide being the main radioiodine metabolite found in the urine after 3 hr. (Albert & Keating, 1951). Subsequently, normal human subjects also were shown to deiodinate [$^{131}I_2$]di-iodotyrosine rapidly (Ruegamer & Chodos, 1956, 1958; Stanbury, Kassenaar, Meijer & Terpstra, 1956). [$^{131}I_2$]Di-iodotyrosine has been found in the plasma (Costa *et al.* 1953) and in the urine (McGirr, Hutchison & Clement, 1956) after administration of ^{131}I in patients with goitrous