CXCVII. METABOLISM OF AMINO-ACIDS.
III. DEAMINATION OF AMINO-ACIDS.

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NEUBAUER [1909; 1928] and Knoop [1925] showed that the deamination of α-amino-acids in the mammalian body is accompanied by the oxidation of the α-C-atom according to the equation

$$\underset{\text{amino-acid}}{R.CHNH_2.COOH} + \tfrac{1}{2}\underset{\text{oxygen}}{O_2} \rightarrow \underset{\text{keto-acid}}{R.CO.COOH} + \underset{\text{ammonia}}{NH_3}. \qquad \ldots\ldots(1)$$

In previous experiments [Krebs, 1933, 1] I have shown that reaction (1) can be conveniently investigated in slices of fresh kidney and liver. It was found that both optical isomerides of the amino-acids are deaminated. In many cases the α-amino-acids which do not occur naturally—belonging to the *d*-series—are deaminated much more rapidly than the "natural" isomerides.

In this paper it will be shown that the enzymic system catalysing the deamination of the natural amino-acids is different from the system catalysing the deamination of the non-natural optical isomerides. Kidney and liver contain (at least) two different enzymic systems responsible for reaction (1). The two systems differ in many ways. The system deaminating the natural amino-acids is destroyed by drying the tissue; it cannot be extracted; it is inhibited by octyl alcohol and by cyanide. The system deaminating the non-natural amino-acids is not destroyed by drying; it is readily soluble in water and can be extracted from fresh or dried tissue by aqueous solutions; it is not affected by octyl alcohol or by cyanide.

I. NOMENCLATURE.

The nomenclature used here for the optical isomerides is that introduced by Fischer [1908] and Wohl and Freudenberg [1923]. The amino-acids belonging to the "natural" series are designated "*l*-amino-acids" on account of their spatial configuration, and members of the "non-natural" series are designated "*d*-amino-acids". The direction of the actual rotation is indicated by (+) or (−).

The system deaminating members of the *l*-series is called "*l*-amino-acid deaminase" and that deaminating members of the *d*-series is called "*d*-amino-acid deaminase".

No evidence exists showing the occurrence of more than one *d*-amino-acid deaminase. All *d*-α-amino-acids seem to be attacked by the same system. But it is possible that more than one *l*-amino-acid deaminase exists.

II. METHODS.

The rate of reaction (1) can be measured by determining either the rate of oxygen uptake or the rate of formation of either keto-acid or ammonia. Only certain preparations of the *d*-amino-acid deaminase however show the strictly stoichiometric proportion expected from equation (1) (see Table VI). In tissue

slices, which are necessary for the investigation of the *l*-amino-acid deaminase, other reactions interfere in which oxygen is absorbed and keto-acids are used up or ammonia is either produced or used up. It is impossible to separate the *l*-amino-acid deaminase from these interfering reactions, and therefore the true rate of reaction cannot be measured. The best approximation is obtained by measuring the rate of formation of ammonia, because the ammonia formation and consumption by other reactions is generally small. The rate of ammonia formation after the addition of amino-acid, corrected for the ammonia produced in the absence of amino-acid, gives a measure of the minimum rate of deamination. On the other hand, the oxygen uptake gives the maximum value. Both values have usually been determined but, when the rate of deamination of *l*-amino-acids is mentioned in this paper, it always refers to the rate of ammonia production.

In the case of the *d*-amino-acid deaminase, where equation (1) is usually fulfilled, the oxygen uptake was generally measured manometrically so that the whole course of the reaction could be followed.

The tissue slice technique and the manometric method have been sufficiently described before [Warburg, 1926]. Open manometers and conical flasks with side-bulbs and inner cups were used. The tissue was suspended in phosphate saline [Krebs, 1933, 1] unless otherwise stated. Substrates were neutralised before being added. The gas space was filled with oxygen; the inner cup contained 0·3 ml. *N* NaOH and filter-paper, according to the technique of Dixon and Elliott [1930].

Ammonia was determined by the method of Parnas *et al.* [1924; 1926; 1934]. The amount of ammonia measured was between 0·01 and 0·1 mg. To make alkaline, 3 ml. of a solution of borax-carbonate were used (50 g. $Na_2B_4O_7$, 10 H_2O+ 10 g. K_2CO_3 (anhydrous) + 1 ml. 0·1 % thymolphthalein per litre). The solution should be faintly blue (p_H 9·5). Under these conditions, the distillation was always complete if 20 ml. of distillate were collected. The distillate was nesslerised and compared with a standard solution. All the reagents used in experiments on ammonia metabolism were tested regularly to see if they were ammonia-free.

The amount of ammonia is expressed as μl. in order to simplify direct comparison with oxygen consumption (17 mg. $NH_3 = 22{,}400\,\mu$l.). The symbols Q_{O_2}, Q_{NH_3} are used for the rate of reaction and mean $\frac{\mu\text{l.}}{\text{mg. hours}}$.

III. Existence of two deaminating systems.

According to Table I, some members of the *d*-series are deaminated by kidney slices much more rapidly than are the corresponding members of the *l*-series.

Table I. *Deamination of* d- *and* l-α-*amino-acids by rat kidney slices.*

[From Krebs, *Z. physiol. Chem.* (1933), **216**, 204, Tables 5 and 6.] The ammonia formation without amino-acid has been subtracted and therefore Q_{NH_3} represents the ammonia formation from the amino-acid. The experimental details are given in the paper quoted.

l-series		*d*-series	
Amino-acid	Q_{NH_3}	Amino-acid	Q_{NH_3}
l(+)Alanine	2·03	*d*(−)Alanine	36·5
l(+)Valine	2·53	*d*(−)Valine	56·5
l(−)Leucine	5·35	*d*(+)Leucine	33·6
l(+)Glutamic acid	7·73	*d*(+)Phenylalanine	75·7
l(−)Aspartic acid	13·9	*d*(+)Aspartic acid	1·26

The existence of two different deaminating systems is shown by the fact that the deamination of *l*-amino-acids is inhibited by conditions which do not affect the deamination of *d*-amino-acids.

(1) *Separation by octyl alcohol.* The substrate used for the *d*-amino-acid deaminase was *d*(−)valine. The *l*(+)valine is only slowly deaminated so that it is not suitable for accurate measurements of the activity of the *l*-amino-acid deaminase. *l*(−)Aspartic acid is the most suitable substrate for this purpose because it shows the highest rate of deamination in the *l*-series. The rate of ammonia formation was estimated as a measure of the rate of deamination. Saturation with octyl alcohol, as shown in Table II, completely inhibits the ammonia formation from *l*(−)aspartic acid.

Table II. *Influence of octyl alcohol (0·01 ml. in 2 ml. fluid) on the deamination of* l(−)*aspartic acid and* d(−)*valine.*

(Slices of rat kidney cortex in 2 ml. phosphate saline. p_H 7·4. 37°. 80 mins.)

Substance added (final concentration)	Q_{O_2}	Q_{O_2} After addition of octyl alcohol	Q_{NH_3}	Q_{NH_3} After addition of octyl alcohol	Q_{NH_3} (corrected for blank)	Q_{NH_3} (corrected for blank) After addition of octyl alcohol
—	−24·1	− 0·5	2·39	2·11		
l(−)Aspartic acid *M*/20	−32·2	− 0·5	9·70	2·22	7·3	0
d(−)Valine *M*/20	−41·4	−33	59·2	70	56·8	68

In the absence of octyl alcohol, the Q_{NH_3} rises from 2·39 to 9·70 after the addition of aspartic acid, but it remains unchanged in the presence of octyl alcohol. With *d*(−)valine, however, octyl alcohol does not depress the ammonia formation. Parallel with the effect of octyl alcohol on the ammonia production is its effect on the oxygen uptake. The oxidation of *l*(−)aspartic acid is inhibited by octyl alcohol, but that of *d*(−)valine is not.

(2) *Separation by extraction.* 1 g. (wet weight) of the rat kidney that was used in the octyl alcohol experiment was ground with sand in a mortar and extracted with 5 ml. of water. The extract was centrifuged and 0·5 ml. of the supernatant fluid was added to three different flasks containing 2 ml. of phosphate saline with (*a*) no amino-acid, (*b*) *M*/20 *l*(−)aspartic acid, (*c*) *M*/20 *d*(−)valine. The other conditions were the same as those given in Table II. The results are shown in Table III.

Table III. *Deamination of* l(−)*aspartic acid and* d(−)*valine by kidney tissue extract.*

Substance added (final concentration)	Oxygen uptake in 80 mins. (μl.)	Ammonia production in 80 mins. (μl.)
—	36	34
l(−)Aspartic acid *M*/20	37	37
d(−)Valine *M*/20	462	930

Grinding and extraction have the same effect as the addition of octyl alcohol, *viz.* complete inhibition of the *l*-amino-acid deaminase and no inhibition of the *d*-amino-acid deaminase. By extraction, or by the addition of octyl alcohol, the action of the *d*-amino-acid deaminase is separated from the action of the *l*-amino-acid deaminase. We have thus the possibility of investigating *d*-amino-acid deaminase free from *l*-amino-acid deaminase. The *l*-amino-acid deaminase, on the

other hand, cannot be separated from the *d*-amino-acid deaminase, but it can be investigated separately if "natural" amino-acids are added to kidney or liver slices. In this case, the presence of the *d*-amino-acid deaminase does not interfere, owing to the absence of substrate.

In the following sections, the properties of the *d*- and *l*-deaminases will be described and their interrelations discussed.

IV. The properties of the *d*-amino-acid deaminase.

(1) *Dry enzyme preparation.* Pig kidney cortex is minced in a "Latapie" mincer within one hour after death. To the minced tissue, 5 vols. of acetone are added. The mixture is thoroughly stirred for 5 mins. and then filtered through a Büchner funnel. The precipitate is dried in a vacuum desiccator over sulphuric acid or phosphorus pentoxide. The dry material is pounded in a mortar. If the powder is kept dry and cool, the activity remains fairly constant for several weeks. After 2 months there was 20–30 % loss in activity.

2 g. of dry powder were shaken for 10 mins. with 80 ml. of water. After being shaken, the fluid was centrifuged. When 2 ml. of the supernatant yellowish fluid (containing 6·2 mg. dry material) were shaken with 0·2 ml. of phosphate buffer (p_H 7·4) at 37° in an atmosphere of oxygen, no oxygen was taken up. When 0·1 ml. of 10 % *dl*-alanine was added, 29 μl. of oxygen were taken up in 10 mins. and 59·5 μl. in 20 mins.

The activity was not increased by prolonged extraction. Extraction with $M/150$ sodium bicarbonate or with $M/150$ KH_2PO_4 gave about the same yield as extraction with water. The insoluble residue was not inactive. When the residue was extracted a second time with 80 ml. of water, an enzyme solution was obtained with about half the activity of the first extract. After 3 or 4 extractions, the insoluble residue was completely inactive. Thus the *d*-amino-acid deaminase is water-soluble.

If slices of kidney cortex are shaken in saline in the presence of oxygen, only small amounts of *d*-amino-acid deaminase pass into the solution. In the absence of oxygen however considerable amounts of the enzyme appear in the solution [Krebs, 1933, 1]. The cells disintegrate more rapidly anaerobically than aerobically and the enzyme can only diffuse out into the solution when the cells are disintegrated.

It is surprising that, although extracting the powder for more than 10 mins. does not increase the activity of the extract, a second extraction for 10 mins. yields a considerable amount of enzyme. This behaviour seems to be due to the fact that, when the extraction is prolonged, inhibiting substances are extracted simultaneously with the enzyme, as shown in the next paragraph.

(2) *Inhibition by tissue substances.* In the previous paper [Krebs, 1933, 1], it was shown that the activity of fresh kidney extracts quickly diminishes: at 37·5°, 30–50 % of the activity was destroyed after 1 hour. Similar observations were made when the dry powder was shaken with amino-acid solution (Table IV and Fig. 1). However, when the powder was extracted for 10 mins. and the extract mixed with amino-acid solution, the activity fell off much more slowly. The stability of the enzyme solution is greater if the insoluble material is removed after brief extraction. The loss of activity is not due to primary instability of the enzyme but to the action of inhibiting substances produced or given off by the tissue material.

The existence of inhibitors can also be demonstrated by diluting the extract (Table IV). The activity of the enzyme is not proportional to the concentration.

Diluted extracts are relatively more active and also the stability of the enzyme increases with dilution. In the example given in Table IV, the loss of activity in the second 40 mins. is 42 % in the undiluted extract and only 15 % in the twice diluted extract.

A direct proof of the presence of inhibiting substances is the inhibition of the enzyme which is observed after the addition of certain tissue extracts. Extracts of rabbit kidney are especially rich in inhibiting substances. 1 ml. of a freshly prepared rabbit kidney extract (1 part of tissue extracted with 40 parts of water) added to 2 ml. of an enzyme preparation from pig kidney (1 part of acetone-dried tissue extracted with 100 parts of water) caused an inhibition of 50 % immediately after the addition, and 80 % after 30 mins. (Table V). Rabbit kidney extract boiled for 10 mins. in a water-bath has no inhibiting power.

A specific inhibition of the enzyme in the intact cell may perhaps account for the results of Abderhalden and Tetzner [1935] who found that rats excrete *d*(−)alanine unchanged.

Table IV. *Oxidation of* dl-*alanine in pig kidney. Comparison of dried powder with extracts from dried powder.*

Extracting fluid *M*/10 phosphate buffer. p_H 7·4.
Time of extraction 10 mins. *M*/30 *dl*-alanine. 37·5°. O_2.

Enzyme preparation	Oxygen uptake (μl.) 10 mins.	20 mins.	40 mins.	60 mins.	80 mins.	100 mins.
50 mg. acetone-dried powder + 3 ml. phosphate buffer	51	91·5	151	187	209	222
3 ml. extract (corresponding to 50 mg. dry powder)	63	118	210	282	332	364
1·5 ml. extract (as above) + 1·5 ml. phosphate buffer	36	67	129	184	230	271

Table V. *Inhibition of* d-*amino-acid deaminase by rabbit kidney extract.*

Total volume of experimental fluid 3·5 ml. *M*/30 *dl*-alanine. *M*/50 veronal buffer. p_H 8·5. 37·5°. O_2.

Enzyme solution	Oxygen uptake (μl.) 10 mins.	20 mins.	30 mins.	40 mins.
2 ml. pig kidney extract	48	87	119	143
1 ml. rabbit kidney extract	0	1	2	3
2 ml. pig kidney extract + 1 ml. rabbit kidney extract	24	41	54	58·5

The following substances, if added, do not affect the oxidation of *dl*-alanine in extracts of dry powder: pyruvate (*M*/100), ammonium chloride (*M*/100), *l*(+)alanine (*M*/50), *d*(−)glutamate (*M*/100), *l*(+)glutamate.

(3) *Ratio ammonia formation/oxygen uptake.* According to equation (1), 2 mols. of ammonia should be formed when 1 mol. of oxygen is taken up. In order to measure this ratio, conical flasks with two side-bulbs were used [Cremer, 1929]. The main compartment of the flask contained the buffered enzyme solution, side-bulb *a* contained 0·1 ml. of *dl*-alanine (*M*), side-bulb *b* 0·1 ml. of 10 % sulphuric acid. The alanine was added to the enzyme after equilibration and after a reading had been taken; the oxygen uptake was then measured over a certain period. Immediately after the last reading, the acid was mixed with the enzyme in order to stop the reaction. The flask was cooled in ice and the ammonia was determined. Blanks without alanine were done in each experiment. The

blank values for oxygen and ammonia were very small in most enzyme preparations and amounted to only a small percentage of the experimental figures.

A number of results are given in Table VI. The figures are corrected for the blank. In many enzyme preparations, the ratio is equal to the theoretical value within the limits of error. In certain preparations however less ammonia is formed than would be expected from the oxygen uptake. In trout kidney, for instance, the ratio is nearly 1:1. Prof. Keilin tells me that he has found that the oxidation of amino-acids under the action of amino-acid deaminase can be coupled with the oxidation of other substances, such as ethyl alcohol. If a suitable substrate is present, an equipartition of oxygen between amino-acid and the

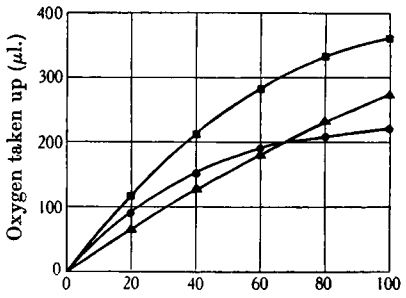

Fig. 1.

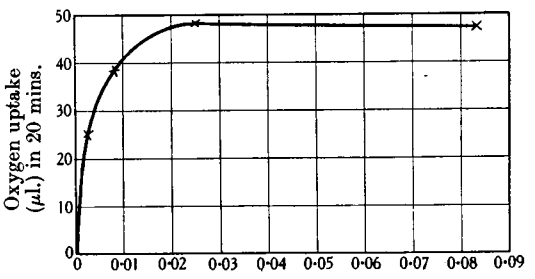

Fig. 2.

Fig. 1. Oxidation of *dl*-alanine in pig kidney. Comparison of dried powder and extracts from dried powder. •—• In presence of powder; ■—■ extract from powder, insoluble residue filtered off; ▲—▲ extract from powder, diluted twice.

Fig. 2. Effect of the concentration of *d*(−)valine on the rate of deamination.

Table VI. *Ratio ammonia formation/oxygen uptake under various conditions.*

M/30 *dl*-alanine (final concentration). 2·0 ml. enzyme solution. 1 ml. buffer. 37·5°. O_2.

Enzyme material	Parts of H_2O used for extraction	Buffer (final concentration)	p_H	Time in mins.	O_2 absorbed (μl.)	NH_3 formed (μl.)	Ratio O_2:NH_3
Pig kidney, acetone preparation	80	Phosphate *M*/30	7·4	30	134	276	1:2·06
,, ,,	80	Veronal *M*/100	8·5	30	260	515	1:1·98
,, ,,	160	Veronal *M*/100	8·5	40	211	422	1:2·00
,, ,,	80	Bicarbonate *M*/100	8·0	30	328	638	1:1·95
Pig kidney, minced, fresh	10	Phosphate *M*/30	7·4	40	246	501	1:2·04
,, (extract kept on ice for 24 hours)	10	Phosphate *M*/30	7·4	30	78	154·5	1:1·98
Rat kidney, minced, fresh	20	Phosphate *M*/30	7·4	40	86	172	1:2·00
Cat liver, minced, fresh	5	Bicarbonate *M*/25	8·5	90	39·2	77·8	1:1·99
Cat kidney, minced, fresh	5	Bicarbonate *M*/25	8·5	90	154	274	1:1·78
Guinea-pig kidney, minced, fresh	5	Phosphate *M*/30	7·4	90	47	84	1:1·77
Pig kidney, minced, fresh	5	Veronal *M*/100	8·5	45	142	254	1:1·79
Trout kidney, minced, fresh	15	Phosphate *M*/30	7·4	40	87	79	1:0·91
Trout liver, minced, fresh	6	Phosphate *M*/30	7·4	40	170	298	1:1·75
Frog (*Rana esculenta*) kidney, minced, fresh	10	Bicarbonate *M*/100	8·0	60	257	493	1:1·92
Frog (*Rana esculenta*) liver, minced, fresh	3	Bicarbonate *M*/100	8·0	60	53	55	1:1·04
Pigeon kidney, minced, fresh	10	Bicarbonate *M*/100	8·0	35	82	101·5	1:1·24
Pigeon liver, minced, fresh	4	Bicarbonate *M*/100	8·0	35	170	298	1:1·75
Newt (*Triton cristatus*) liver, minced, fresh	20	Phosphate *M*/100	7·4	60	47	80	1:1·70
Tortoise (*Testudo graeca*) liver, minced, fresh	5	Phosphate *M*/100	7·4	40	68	79	1:1·16
Tortoise kidney, minced, fresh	10	Phosphate *M*/100	7·4	60	26	46	1:1·77

other substrate takes place. The ratio ammonia formation/oxygen uptake is thus reduced from 2 to 1. Most enzyme preparations lack a suitable substrate for the coupled reaction or lack the catalyst necessary for the coupling. The ratio ammonia formation/oxygen uptake is in these cases that expected from equation (1). If substrate and catalyst are present, the ratio decreases. Keto-acids are not oxidised by any of the enzyme preparations tested and therefore the decrease in the ratio cannot be due to an oxidation of the amino-acid beyond the stage of the keto-acid.

(4) *Influence of substrate concentration.* 1 g. of acetone-dried pig kidney was extracted with 20 ml. of water by shaking for 10 mins. To 1 ml. of the supernatant fluid, 1 ml. of phosphate buffer (p_H 7·4, $M/10$) and 1 ml. of *d*(−)valine in various concentrations were added. The oxygen uptake was measured manometrically. Oxygen pressure 1 atmosphere. 37·5°. The results are given in Table VII and Fig. 2.

Table VII. *Influence of the* d(−)*valine concentration on the rate of oxidation.*

Concentration of *d*(−)valine (mols. per litre)	Oxygen uptake in 20 mins. (μl.)
0·083	47·4
0·025	48·4
0·0083	38·5
0·0025	25·0

The maximum rate is reached when the concentration of the substrate is about $0{\cdot}2M$. Half the maximum rate occurs at the concentration $0{\cdot}002M$.

(5) *Influence of the oxygen pressure.* 1 g. of acetone-dried pig kidney was extracted with 20 ml. of water. To 2 ml. of the extract, 0·1 ml. of *dl*-alanine (M) and 0·2 ml. of phosphate buffer ($M/10$, p_H 7·4) were added. The oxygen uptake in air (37·5°) was 52·9 μl. after 30 mins., 99·4 μl. after 60 mins. In oxygen at 1 atmosphere pressure, the oxygen uptake was 59·1 μl. after 30 mins., 112·0 μl. after 60 mins. Thus the increase of oxygen pressure from 0·21 atm. to 1 atm. increases the rate of oxidation by 11 %. The shaking was so fast that further increase of its speed did not influence the results.

(6) *Influence of inorganic salts.* $M/10$ sodium chloride inhibits the activity of an aqueous enzyme solution by about 25 %, M sodium chloride by 85 %. Stronger concentrations of neutral salts or buffers should therefore be avoided in the investigation of the *d*-amino-acid deaminase.

(7) p_H *optimum.* The p_H curve of the *d*-amino-acid deaminase shows a rather sharp optimum near p_H 8·8 (Table VIII, Figs. 3 and 4). In alkaline solutions the activity of the enzyme diminishes rapidly, whereas near the neutral point the activity remains fairly constant for a considerable period. *dl*-Alanine and *dl*-aspartic acid give similar p_H curves with the same maximum. Sparingly soluble amino-acids such as cystine or tyrosine are only slowly oxidised at p_H 7·4, but undergo rapid oxidation at p_H 8·5 where the solubility is markedly greater.

(8) *Influence of cyanide.* $M/10$ cyanide has no influence on the oxidation of alanine by the *d*-amino-acid deaminase (p_H 7·4; concentration of *dl*-alanine M or $M/100$).

(9) *Influence of narcotics.* Octyl alcohol in saturated solution does not inhibit the *d*-amino-acid deaminase as shown in Table II. Chloroform and toluene are likewise without action.

(10) *Reduction of methylene blue.* Bernheim and Bernheim [1934; 1935] have shown that amino-acids added to kidney extracts reduce methylene blue; they

Table VIII. p_H *optimum of the oxidation of* dl-*alanine and of* dl-*aspartic acid in presence of* d-*amino-acid deaminase.*

(*a*) *dl*-alanine: each flask contained 2 ml. of enzyme solution (1 g. pig kidney acetone-dried powder extracted with 40 ml. of water), 1 ml. of buffer and 0·1 ml. *dl*-alanine (*M*). 37°. O_2.

Buffer added	p_H	Oxygen uptake (μl.) 10 mins.	20 mins.
Carbonate-bicarb. (*M*/5)	10·8	−2·5	4
,,	9·8	70	111
,,	8·8	110	203
Phosphate (*M*/10)	7·4	40	78
,,	6·8	19	38

(*b*) *dl*-aspartic acid: each flask contains 2 ml. enzyme solution, 0·5 ml. of *M*/5 *dl*-aspartic acid (neutralised) and 1 ml. of buffer. Other conditions as above.

Buffer	p_H	Oxygen uptake (μl.) 20 mins.	40 mins.
Carbonate-bicarb. (*M*/5)	10·8	16	30·5
,,	9·8	38	72
Veronal (*M*/10)	9·4	49	99
,,	8·4	50	100
,,	7·4	29	58
,,	6·3	8	15

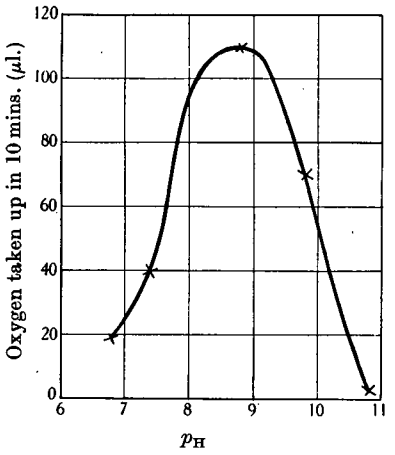

Fig. 3. p_H curve of the *d*-amino-acid deaminase. Substrate *dl*-alanine.

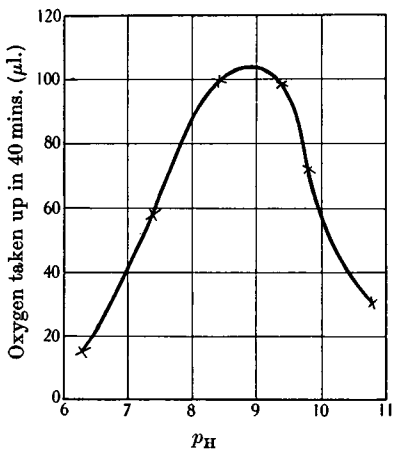

Fig. 4. p_H curve of the *d*-amino-acid deaminase. Substrate *dl*-aspartic acid.

found that *dl*-leucine reduces methylene blue faster than *dl*-alanine. With *dl*-alanine I find no change in the reduction time as compared with the blank. With *dl*-leucine however a reduction of methylene blue was observed, in accordance with the observation of Bernheim and Bernheim, and ammonia was formed. As enzyme material, an aqueous extract (50 parts of water) of acetone-dried pig kidney (1 part) was used. The enzyme solution and buffer were measured into the main compartment of a conical Warburg flask. The methylene blue (0·1–0·2 ml.) and amino-acid solutions were placed in the side-bulb. The inner cup contained Fieser's [1924] $Na_2S_2O_4$ solution for absorbing contaminating oxygen. The gas space was filled with nitrogen. After equilibration for 20 mins., the amino-acid and methylene blue were tipped on to the enzyme. The meniscus of the manometer did not change in the following period, showing that no gas was taken up.

This excludes the possibility that the ammonia found was due to the action of contaminating oxygen. A high concentration of methylene blue was used (1 or 2 mg. in each flask). Thus the total amount of ammonia was large enough for accurate determination. The results of the experiment are given in Table IX.

Table IX. *Reduction of methylene blue by* dl-*leucine.*

37·5°. Details in text.

Enzyme solution, buffer, p_H	Amount of methylene blue added (millimols.)	Final concn. of *dl*-leucine	Time in mins.	State of methylene blue at the end	NH_3 formed (μl.)
4 ml. kidney extract; 1 ml. *M*/10 phosphate buffer; p_H 7·4	$2·75 \times 10^{-3}$	0	110	Almost unchanged	30·5
,, ,,	$2·75 \times 10^{-3}$	*M*/80	110	Completely colourless	69
5 ml. kidney extract; 1 ml. *M*/10 phosphate buffer; p_H 7·4	$5·5 \times 10^{-3}$	0	130	Almost unchanged	41
,, ,,	$5·5 \times 10^{-3}$	*M*/160	130	Completely colourless	114
5 ml. kidney extract; 0·3 ml. *M*/10 veronal buffer; p_H 8·5	$5·5 \times 10^{-3}$	0	90	Almost unchanged	41
,, ,,	$5·5 \times 10^{-3}$	*M*/160	90	Completely colourless	112

The experiments show that in reaction (1) molecular oxygen can be replaced, in certain cases, by methylene blue. The rate of reaction in the case of leucine is about 50 times slower with methylene blue than with molecular oxygen. The difference is still greater with other amino-acids.

The ammonia formed amounts only to about half the quantity calculated for the amount of methylene blue reduced. This may be explained by partial reduction of methylene blue by keto-acids or other substances present in the enzyme preparations.

(11) *Occurrence in different tissues.* The *d*-amino-acid deaminase occurs in the liver and kidney of all the vertebrates investigated (Tables VI, X). Intestinal wall, spleen, muscle, brain, testis, placenta, chorion, retina, salivary glands, pancreas and heart of the rat do not contain measurable amounts of the enzyme. According to Table X, kidney is about 4 times as active as liver. Acetone-dried

Table X. d-*Amino-acid deaminase in liver and kidney.*

Material	Oxygen uptake after addition of *dl*-alanine in 20 mins. 37·5°. O_2 (μl.)
Pig liver	9
Pig kidney	36
Sheep liver	39
Sheep kidney	152

powder of liver and kidney cortex, prepared as described, were extracted with 50 parts of water for 10 mins. To 2 ml. of the supernatant fluid, 0·2 of phosphate buffer (*M*/10, p_H 7·4) and 0·1 ml. of *dl*-alanine (*M*) were added and the results given in Table IX were obtained. In the example given, sheep tissues were about 4 times as active as pig tissue but this is not a regular difference. The activities of sheep and pig tissue are, on the average, of the same order of magnitude.

(12) *Final value of oxygen uptake.* In most preparations the oxygen uptake in the presence of *dl*-alanine corresponds exactly to the theoretical amount calculated on the assumption that only one optical component is oxidised according to equation (1). 4 mols. of *dl*-alanine give rise to the uptake of 1 mol. of oxygen (Table XI and Fig. 5).

Table XI. *Final value of oxygen uptake.*

1 g. of sheep kidney acetone-dried powder extracted with 12·5 ml. of water. The flask contained 2 ml. of enzyme solution and 0·2 ml. of phosphate buffer ($M/10$, p_H 7·4) in the main compartment and 0·20 ml. of *dl*-alanine (0·099 M) in the side-bulb. The alanine was added after equilibration. 37·5°. O_2. Theoretical oxygen uptake 111 μl.

Time after addition of alanine (mins.)	Oxygen uptake (μl.)
5	52
10	84
20	104
40	108
80	108

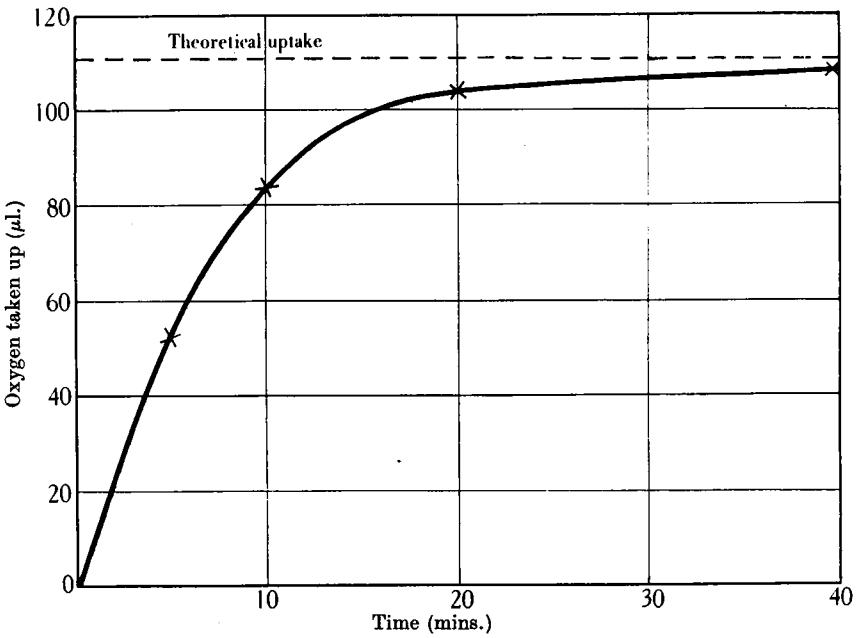

Fig. 5. Final value of the oxygen uptake of *dl*-alanine.

(13) *Oxidised amino-acids.* 1 g. of acetone-dried pig kidney was shaken for 10 mins. with 40 ml. of water. 2 ml. of extract (containing 6·8 mg. of dry material), 0·2 ml. of M $NaHCO_3$ and 0·3 ml. of amino-acid solution ($M/3$ in the case of the *dl*-compounds, $M/6$ in the case of the *d*-compound) were placed in a manometric flask. If the amino-acid were sparingly soluble, the equivalent amount was added as powder. The p_H was 8·5 (colorimetrically). The oxygen uptake was measured at 37·5° in oxygen. The inner cup of the flask contained 0·3 ml. of 2N NaOH and filter-paper. Table XII shows the amino-acids which are deaminated in the presence of *d*-amino-acid deaminase and the rate of oxidation.

Table XII. *Rate of oxidation of various amino-acids in the presence of* d-*amino-acid deaminase.*

For experimental conditions see text. B.D.H. = British Drug Houses. H.L.R. = Hoffmann-La Roche. F.L. = Fränkel and Landau, Berlin-Oberschönweide.

Amino-acid	Oxygen uptake (μl.) in 10 mins.	Oxygen uptake (μl.) in 20 mins.	Preparation of amino-acid used
dl-Alanine	72	138	B.D.H.
dl-α-Aminobutyric acid	29	55	F.L.
dl-*nor*Valine	25	53	F.L.
d(−)Valine	59	106	H.L.R.
dl-*nor*Leucine	62·5	121	F.L.
dl-Leucine	27·5	55	F.L.
d-*iso*Leucine	93	176	H.L.R.
dl-α-Aminocaprylic acid	4·6	11	F.L.
dl-Serine	43	78	H.L.R.
dl-α-Phenyl-α-amino-acetic acid	18·5	35·5	F.L.
d(−)Phenylalanine	87	143	H.L.R.
dl-Tyrosine	27	51	Prepared by Dr N. W. Pirie
dl-Tryptophan	9·5	19	Prepared according to du Vigneaud *et al.* [1932, 1, 2]
d(+)Histidine	7	16·5	H.L.R.
dl-Aspartic acid	8·6	15	F.L.
dl-Glutamic acid	3	5	Prepared according to Abderhalden and Kautsch [1910]
dl-β-Hydroxyglutamic acid	0	0	Gift from Prof. C. R. Harington [Harington and Randall, 1931]
dl-α-Amino-β-γ-dihydroxy-butyric acid	3·5	9	Gift from Prof. H. O. L. Fischer [Fischer and Feldmann, 1932]
dl-Arginine	24	41	Prepared after Bergmann and Köster [1926]
dl-Cystine	34	54	Prepared by Dr Pirie
dl-Methionine	139	269	Prepared by Dr Pirie
dl-*S*-Ethylcysteine	80·5	131	Prepared by Dr Pirie [see Clarke and Inouye, 1931]

No *d*- or *dl*-α-amino-acid has been found—with the exception of *dl*-β-hydroxyglutamic acid—which is not attacked by the enzyme. The velocity of the oxidation varies with the chemical structure. Some sulphur-containing amino-acids (*dl*-methionine, *dl*-*S*-ethylcysteine) show the highest rate of oxidation. The simple α-amino-acids (alanine, valine, leucine) follow next. The oxidation of *dl*- or *d*(−)glutamic acid is slow. *d*(−)Ornithine and *d*(−)lysine have not yet been examined. None of the amino-acids of the *l*-series is oxidised. Inactive glycine is not attacked, whereas phenylglycine, which has an asymmetric C-atom, is oxidised. Amino-acids in which the amino-group is not in the α-position (*dl*-β-alanine, *dl*-β-aminobutyric acid, *dl*-ε-amino-*n*-hexanoic acid) are not oxidised, nor are the dipeptides, *dl*-alanylglycine and *dl*-leucylglycine.

The specificity of the enzyme may be used for the determination of the optical configuration of an amino-acid. It may also be used for resolving *dl*-amino-acids and for preparing the *l*-component, thus supplementing Ehrlich's [1906; 1914; 1927] yeast method which yields the *d*-component.

(14) *Occurrence of* d-*amino-acids.* It seems strange that an enzyme exists that deals specifically with non-natural substrates. But it may be pointed out that α-amino-acids of the *d*-series have been found occasionally in nature (for reference see Ehrlich [1914], v. Lippmann [1884], Fränkel *et al.* [1923; 1924]). The enzyme described in this paper may be a helpful reagent for tracing

d-amino-acids. By adding kidney extract to a solution and measuring the oxygen uptake and ammonia formation, *d*-amino-acids can be detected in the solution.

(15) *Behaviour of the* d-*amino-acid deaminase in tissue slices.* The experiments dealt with in the preceding paragraphs were carried out on enzyme preparations of tissue, *i.e.* aqueous extracts of dried or fresh tissue. When slices instead of extracts were used as enzyme material, certain properties of the system were found to be different. Unlike the extracted enzyme, the system in the intact cell is inhibited by cyanide and by oxidisable substances.

(*a*) *Inhibition by cyanide.* The inhibition by cyanide of the *d*-amino-acid deaminase in slices is less than the inhibition of cell respiration. Under the same conditions, the respiration of kidney is inhibited 80 % by $10^{-3}M$ cyanide and 90 % by $10^{-2}M$ cyanide. The oxidation of *dl*-alanine is inhibited about 10 % and 40 % by 10^{-3} and $10^{-2}M$ cyanide respectively (Table XIII).

Table XIII. *Inhibition of* d-*amino-acid deaminase by HCN in tissue slices.*

Concentration of *dl*-alanine 0·05 *M*. O_2.

Tissue	Medium	Temperature	Time in mins.	Concentration of HCN *M*	Q_{NH_3}	% inhibition
Sheep kidney	Bicarbonate saline, 5 % CO_2 in O_2	24°	70	0	3·24	—
		24°	70	10^{-3}	3·02	8
		24°	70	10^{-2}	2·03	37
Rat kidney	,,	37·5°	70	0	26·6	—
	,,	37·5°	70	10^{-3}	24·2	9
	,,	37·5°	70	10^{-2}	15·4	42
Rat kidney	Phosphate saline	37·5°	60	0	18·6	—
	,,	37·5°	60	10^{-3}	14·7	21
	,,	37·5°	60	10^{-2}	8·1	56
Rat kidney	,,	20°	60	0	3·54	—
	,,	20°	60	10^{-3}	3·38	5
	,,	20°	60	10^{-2}	2·37	33

The determination of ammonia in the presence of cyanide requires special precautions because cyanide prevents the formation of the yellow colour with Nessler's reagent. If borate buffer is used for liberating ammonia, hydrocyanic acid is found in the distillate. In the presence of cyanide, therefore, stronger alkali must be used. When the amount of cyanide is 3 ml. of *M*/100, 2 ml. of 2*N* NaOH prevent the appearance of significant amounts of cyanide in the distillate.

Similar differences in the inhibition of oxidations in intact cells and in extracts have been found before by Alt [1930] and by Warburg [1931].

In order to explain the fact that cyanide acts differently in slices and in extracts, I follow Warburg and Christian [1931; 1932] and assume that the mechanism of the oxidation of *dl*-alanine is different in slices and in extracts. In extracts, oxygen may react more or less directly with the activated substrate. In slices, oxygen may be prevented from reacting directly with the activated substrate and an activation of oxygen may be necessary; this process is known to be inhibited by cyanide. The view that molecular oxygen does not react in the intact tissue in the same way as in the extract is supported by the finding of inhibiting substances in the tissue which prevent the reaction between oxygen and the amino-acid (Section IV, 2).

(*b*) *Inhibition by oxidisable substances.* The ammonia production from *dl*-alanine (*M*/50) is inhibited about 50 % by *M*/25 *dl*-lactate (Table XIV). Both alanine and lactate cause a large increase in oxygen uptake if added to kidney.

Table XIV. *Inhibition of ammonia production from* dl-*alanine by lactate.*

Rat kidney slices in phosphate saline. O_2.

Substrate added (final concentration)	Temperature	Q_{O_2}	Q_{NH_3}
—	25°	− 9·30	1·18
M/75 *dl*-lactate	25°	−15·2	0·95
M/30 *dl*-alanine	25°	−19·7	8·00
M/75 *dl*-lactate + *M*/30 *dl*-alanine	25°	−19·1	3·72
—	37·5°	−27·1	3·00
M/75 *dl*-lactate	37·5°	−45·4	2·05
M/30 *dl*-alanine	37·5°	−73·5	50·5
M/75 *dl*-lactate + *M*/30 *dl*-alanine	37·5°	−60·0	25·2
—	21°	− 5·00	—
M/100 *dl*-lactate	21°	−11·3	0·34
M/30 *dl*-alanine	21°	−16·4	8·14
M/100 *dl*-lactate + *M*/30 *dl*-alanine	21°	−16·2	5·12

If however the two substances are added together, no summation of the increasing effect on oxygen uptake is observed. The experiments carried out at 25° or 21° are especially convincing since at these temperatures diffusion is certainly not the limiting factor in respiration. The non-summation of the oxygen uptake suggests that the inhibition of ammonia production is due to the fact that, in the presence of lactate, oxygen is partly used for the oxidation of lactate, in other words lactate and alanine compete for the activated oxygen. This would imply that the activation of oxygen is, in its first step, identical for the oxidation of *d*-alanine and *dl*-lactate. This problem will be discussed fully in a later section of this paper.

V. The properties of the *l*-amino-acid deaminase.

(1) *Destruction by drying, extraction or narcotics.* Unlike the *d*-amino-acid deaminase, the *l*-enzyme is destroyed by drying or extracting the ground tissue (see Table III) or by addition of octyl alcohol (see Table II). The *l*-amino-acid deaminase is "bound up with the structure of the living cell". For the investigation of the enzyme therefore tissue slices provide the most suitable material. In slices, the activity of the enzyme remains fairly constant for 2–3 hours.

(2) *Inhibition by cyanide.* The inhibition of the *l*-amino-acid deaminase by cyanide is of the same order of magnitude as the inhibition of the cell respiration, (Table XV). $10^{-4}M$ cyanide inhibits the ammonia formation from *l*-aspartic acid by 89 %, $10^{-3}M$ inhibits by 94 %. (KCN must be tested for freedom from ammonia which is often present in old solutions.)

In experiments with cyanide, special precautions have to be observed if the inner cup of the manometric flask contains alkali for the absorption of carbon dioxide. Alkali rapidly absorbs hydrocyanic acid. If the concentration of the cyanide is small ($10^{-4}M$), all the hydrocyanic acid distills over into the inner cup within a short period and no inhibition ensues. If the concentration of cyanide is high, errors arise because the pressure of the HCN gradually decreases owing to absorption. These errors can be avoided if the inner cup contains an alkali-cyanide mixture, in which the concentration of free HCN is equal to the con-

centration in the experimental fluid. In the presence of HCN therefore the following solutions were used:

Concentration of HCN in the experimental fluid (mol./litre)	Absorbing solution in inner cup
10^{-2}	10 ml. 2*N* KCN + 0·2 ml. *N* KOH
10^{-3}	10 ml. *N* KCN + 1 ml. *N* KOH
10^{-4}	5 ml. *N* KCN + 5 ml. *N* KOH
10^{-5}	1 ml. *N* KCN + 10 ml. *N* KOH

The concentrations of free hydrocyanic acid in the absorbing fluid, calculated from the data of Walker [1889] are roughly the same as in the experimental fluid.

Table XV. *Inhibition of the deamination of* l-*aspartic acid by hydrocyanic acid.*

Rat kidney cortex slices in phosphate saline (3 ml. in each flask). 37·5°. O_2.

Concentration of *l*(−)aspartic acid	HCN *M*	Q_{O_2}	Inhibition of oxygen uptake by HCN %	Q_{NH_3}	Extra ammonia after addition of *l*(−)aspartic acid per mg. and hour	Inhibition of the deamination of *l*(−)aspartic acid by HCN %
0	0	−19·2	—	1·40	—	—
M/5	0	−27·7	—	8·38	6·98	—
0	10^{-4}	− 4·92	77	1·38	—	—
M/5	10^{-4}	− 8·70	69	2·43	0·95	89
0	10^{-3}	− 2·53	87	1·02	0	—
M/5	10^{-3}	− 2·68	90	1·49	0·47	94

(3) p_H *optimum curve.* In order to obtain different hydrogen ion concentrations, the concentrations of bicarbonate and free carbon dioxide were varied in the medium (Table XVI). The deamination of *l*-aspartic acid shows an optimum (Fig. 6) at the physiological p_H (7·4). It falls off steeply towards the alkaline side, but slowly towards the acid side. At p_H 6·5, the deamination is still 60 % of the maximum rate. Within the range of p_H occurring physiologically, there is no

Table XVI. *Influence of* p_H *on the deamination of l*(−)*aspartic acid in rat kidney cortex.*

Medium; isotonic saline with varying concentrations of bicarbonate and carbon dioxide. The solutions were prepared by mixing bicarbonate-free saline with isotonic (0·155*M*) sodium bicarbonate. Concentration of *l*(−)aspartic acid *M*/50. 37·5°. Duration of experiment: 1 hour.

Concentration of bicarbonate (mol./litre)	0·155	0·155	0·053	0·026	0·0078	0·0026	0·0026	0·00078
Concentration of CO_2 (mol./litre)	0·0005	0·0011	0·0011	0·0011	0·0011	0·0011	0·0045	0·00045
Concentration of CO_2 (vols. % of gas mixture)	2·5	5·0	5·0	5·0	5·0	5·0	20·0	20·0
p_H	8·57	8·28	7·82	7·40	6·96	6·49	5·18	5·36
Q_{NH_3} (in the presence of aspartic acid)	1·41	2·44	8·56	14·1	12·9	8·8	7·6	4·72
Q_{NH_3} in the control in absence of aspartic acid	1·26	1·32	1·38	1·47	1·03	1·31	1·11	1·68
Q_{NH_3} (in the presence of aspartic acid, corrected for blank)	0·15	1·12	7·18	12·6	11·9	7·5	6·5	3·0

considerable difference in the velocity of ammonia formation. If no substrate is added, the ammonia formation changes only slightly with p_H (see also Patey and Holmes [1930]).

The kidney excretes the more ammonia the more acid the urine or the smaller the concentration of bicarbonate in the blood [Hasselbalch, 1915]. Thus the excretion of ammonia does not run parallel with its formation from amino-acids. From the work of Nash and Benedict [1921] we know that renal veins contain much more (up to 20 times) ammonia than the renal artery. This indicates that normal kidney produces more ammonia than is needed for excretion. It depends on the conditions of the acid-base equilibrium whether the ammonia formed in kidney from amino-acids is excreted in the urine or appears in the venous blood.

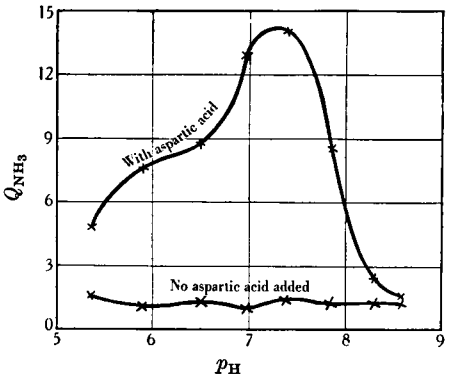

Fig. 6. p_H curve of the *l*-amino-acid deaminase. Substrate *l*(−)aspartic acid. Rat kidney.

(4) *Influence of substrate concentration.* *M*/200 *l*-aspartic acid gives almost the maximum rate of deamination (Table XVII). At lower concentrations of substrate, the ammonia formation is considerably smaller. It may be noted that the amino-nitrogen concentration of blood plasma lies between *M*/100 and *M*/400 [Van Slyke and Meyer, 1912; 1913]. The concentration curve is modified by the presence of other oxidisable substances, as shown in Table XVIII. This phenomenon will be discussed in Section V, 7.

Table XVII. *Influence of substrate concentration on the deamination of* l(−)*aspartic acid.*

Rat kidney. Phosphate saline. 1 hour. 37·5°.

Concentration of *l*(−)aspartic acid *M*	Q_{O_2}	Q_{NH_3}
0·04	−23·8	9·6
0·02	−30·1	12·4
0·01	−34·2	11·1
0·005	−32·1	10·6
0·0025	−28·6	6·42
0	−21·5	1·97

Table XVIII. *Influence of the concentration of* l(−)*aspartic acid on the ammonia formation in presence of* M/*100* dl-*lactate.*

Rat kidney. Phosphate saline. 1 hour.

Concentration of *l*(−)aspartic acid *M*	Q_{O_2}	Q_{NH_3}
0·07	−40·2	9·10
0·035	−44·1	6·06
0·0175	−48·0	5·30
0·0088	−50·2	3·43
0·0044	−46·0	2·21
0·0022	−49·2	1·32
0	−44·0	1·15

(5) *Reduction of methylene blue.* Tissue slices of kidney slowly reduce methylene blue if kept in saline at 37·5° in the absence of oxygen. For instance, 3 mg. of methylene blue in 6 ml. saline were reduced by 44 mg. (dry weight) rat kidney slices in 2 hours. Addition of $M/100$ *l*(−)aspartic acid did not influence the velocity of reduction nor did it increase ammonia formation. Brilliant cresyl blue is reduced a little more quickly than methylene blue but again aspartic acid has no effect on the rate of ammonia production. It should be emphasised that the non-reduction of these indicators does not prove anything as to the activation or non-activation of the substrate molecule.

Thunberg [1920; 1923] has studied the reduction of methylene blue by a large number of amino-acids, but none except glutamic acid and perhaps alanine gave increased reduction in muscle or nervous tissue. The question whether the reduction of methylene blue in the presence of glutamic acid is accompanied by formation of ammonia was not investigated.

(6) *Separation of the* l*-amino-acid deaminase from cell respiration.* The *l*-enzyme resembles the general cell respiration as regards sensitivity to drying, extraction, octyl alcohol or hydrocyanic acid. It differs however from most other cellular oxidations as to sensitivity to arsenious oxide. Arsenious oxide strongly inhibits cell respiration [Warburg and Onaka, 1911]; it inhibits particularly the breakdown of the keto-acids formed according to equation (1) [Krebs, 1933, 1, 2]. It does not inhibit the deamination of *l*(−)aspartic or *l*(+)glutamic acid. In the presence of arsenious oxide therefore keto-acids accumulate in the solution. Aspartic acid yields oxaloacetic and pyruvic acids, glutamic acid yields α-ketoglutaric acid. The keto-acids can be detected by carboxylase or can be isolated as dinitrophenylhydrazones [Krebs, 1933, 2]. Thus kidney is able to split off the ammonia from *l*(−)aspartic and *l*(+)glutamic acids according to equation (1) without oxidising the rest of the amino-acid molecule. We may therefore speak of a *l*-amino-acid deaminase as an oxidising system different from the other systems in the tissue.

(7) *Inhibition by oxidisable substances.* The deamination of *l*-amino-acids is inhibited if substances which are readily oxidised, such as lactate or pyruvate or succinic acid or α-keto-acids are added. Some examples are given in Table XIX.

Table XIX. *Inhibition of ammonia production from* l(−)*aspartic acid by oxidisable substances.*

Rat kidney. Concentration of the substrates $M/100$. 37·5°.

Substrates added	Q_{O_2}	Q_{NH_3}
No substrate	−21·0	1·52
l(−)Aspartic acid	−37·1	12·6
Pyruvic acid	−42·2	—
Lactic acid	−40·9	—
l(−)Aspartic + pyruvic acids	−39·1	4·56
l(−)Aspartic + *dl*-lactic acids	−38·5	4·21
l(+)Glutamic acid	−41·0	6·03
α-Ketoglutaric acid	−38·4	0·34
l(+)Glutamic + α-ketoglutaric acids	−42·0	0·94

Addition of $M/100$ *dl*-lactate to $M/100$ *l*(−)aspartic acid inhibits ammonia formation by 67 %. $M/100$ ketoglutaric acid inhibits ammonia production from $M/100$ *l*(+)glutamic acid almost completely. Glucose has no appreciable effect on the oxidation of amino-acids.

This inhibition is not due to secondary utilisation of ammonia in the presence of the added substrate since added ammonia disappears very slowly from rat

Table XX. *Utilisation of ammonia in rat kidney in the presence of various substrates.*

Substrate added	mg. tissue	Amount of ammonia (μl.) in 3 ml. of solution: Initial	Amount of ammonia (μl.) in 3 ml. of solution: After 80 mins.	Q_{NH_3}	Q_{O_2}
—	8·85	29·2	35·5	+0·56	−26·1
dl-Lactate (*M*/100)	10·96	29·2	23·7	−0·38	−40·5
Pyruvate (*M*/100)	11·69	29·2	24·2	−0·32	−41·2
α-Ketoglutaric acid (*M*/100)	9·99	29·2	23·5	−0·43	−37·2

kidney in the presence of these substrates (Table XX). If the tissue is offered several substrates, they compete for the oxygen available. Each of these substrates increases the Q_{O_2} from about 20 to about 40 (37·5°). In the presence of several substrates however no summation of the increasing effect on respiration ensues; the oxygen uptake is again about 40. The constant oxygen uptake, together with the decreased ammonia production observed when a second substrate is added to amino-acids, indicates that the second substrate is oxidised instead of the amino-acid ("sparing action", see also Dickens and Greville [1933]).

In order to ensure that a maximum figure of Q_{O_2} is not limited by the rate of the diffusion, some experiments were carried out at 20°, where the velocity of reactions is less and diffusion cannot be a limiting factor. Again no summation was found but there was displacement of amino-acid by other substrates (Table XXI).

Table XXI. *Inhibition of ammonia production from* l(+)*glutamic acid by α-ketoglutaric acid at 20°.*

	Rat kidney.	
Substrates added (*M*/100)	Q_{O_2}	Q_{NH_3}
—	−3·82	0·36
l(+)Glutamic acid	−6·68	1·29
α-Ketoglutaric acid	−6·52	0·23
l(+)Glutamic acid + α-ketoglutaric acid	−7·50	0·13

I explain the fact that no summation takes place by assuming that the mechanism of activation of molecular oxygen is identical when different substrates are oxidised. But the following experiments make it probable that the activation of the substrate is different in the case of *l*-amino-acids and other substrates. If arsenious oxide ($10^{-3}M$) is added to kidney, the oxidation of α-keto-acids is more inhibited than the oxidation of amino-acids. Arsenious oxide therefore can restore the oxidation of amino-acids which was inhibited by the presence of keto-acids (Table XXII). If amino-acids compete with other substrates for oxygen, arsenious oxide shifts the position in favour of the oxidation of amino-acids. The simplest explanation of this effect is the assumption that the activation of amino-acids is less inhibited by arsenic than is the activation of other substrates.

The inhibition of cellular oxidation by arsenious oxide was discovered by Warburg and Onaka [1911]. The experiments of Banga *et al.* [1931] have already suggested that the partial reaction affected is the activation of the substrate (dehydrogenase).

Table XXII. *Influence of As_2O_3 on oxygen uptake and ammonia production.*

Rat kidney. 25°.

	Substrates added	As_2O_3 concentration M	Q_{O_2}	Q_{NH_3}
1.	—	0	− 8·50	0·63
2.	—	10^{-3}	− 2·00	0·75
3.	*l*(+)Glutamic acid (*M*/100) + α-ketoglutaric acid (*M*/100)	0	−18·7	0
4.	*l*(+)Glutamic acid (*M*/100) + α-ketoglutaric acid (*M*/100)	10^{-3}	− 3·42	1·13

The inhibition of deamination by lactate described in this paragraph explains why kidney slices may produce less ammonia if suspended in serum than in saline. It may also explain why in perfusion experiments the rate of deamination in kidney was found to be small or negligible [Bornstein and Budelmann, 1930]. Under the conditions of a perfusion experiment, the lactic acid concentration in blood is often unphysiologically high (owing to blood glycolysis, effect of anaesthetics and various other circumstances).

The method of investigating the summation offers the possibility of deciding generally whether the oxidation of various substances in cells involves entirely separate systems for each substrate. Some experiments (Table XXIII) were

Table XXIII. *Oxygen uptake of baker's yeast in the presence of various substrates.*

Each flask contained 3 ml. of yeast suspension (4 mg. dry weight) in *M*/20 KH_2PO_4. 17°.

Substrate added (final concentration)	Oxygen uptake (μl.) 40 mins.	80 mins.	Q_{O_2}
—	21	34	− 6·4
Glucose (*M*/20)	224	435	−81·5
Sodium *dl*-lactate (*M*/50)	188	388	−72·6
Sodium acetate (*M*/20)	67	199	−37·5
Sodium butyrate (*M*/20)	62	116	−21·8
Sodium α-ketoglutarate (*M*/50)	164	342	−62·1
Glucose (*M*/20) + sodium *dl*-lactate (*M*/50)	218	435	−81·5
Glucose (*M*/20) + sodium acetate (*M*/20)	212	432	−81·0
Glucose (*M*/20) + sodium butyrate (*M*/20)	192	402	−75·2
Glucose (*M*/20) + sodium α-ketoglutarate (*M*/50)	245	426	−80·0

carried out with yeast cells. Yeast is a very suitable material since its respiration without substrate is very small and the addition of substrate causes a great increase (more than 1000 %) in the respiration. Suitable substrates of different types are glucose, *dl*-lactate, acetate, butyrate and α-ketoglutarate. Each substrate brings about a large increase in respiration, but no summation ensues when two substrates are added simultaneously. This seems to prove that the systems responsible for the oxidation of various substrates have one component in common, most probably the part which activates the molecular oxygen.

(8) *Occurrence of* l*-amino-acid deaminase in different tissues.* Kidney cortex shows by far the highest rate of deamination of *l*-amino-acids in all the animals examined (rat, guinea-pig, rabbit, cat, dog, sheep, pig). Table XXIV gives some instances comparing the oxygen uptake and the ammonia production in liver and kidney after the addition of *l*(−)aspartic acid. In kidney the amino-acid roughly doubles the oxygen uptake and causes a large increase in the ammonia

Table XXIV. *Oxygen uptake and ammonia formation of various tissues in the presence of* l(−)*aspartic acid.*

Tissue	Oxygen uptake (Q_{O_2})		Ammonia formation (Q_{NH_3})	
	Without l(−)aspartic acid (*M*/5)	With l(−)aspartic acid (*M*/5)	Without l(−)aspartic acid (*M*/5)	With l(−)aspartic acid (*M*/5)
Kidney, rat	−19·2	−27·7	1·40	8·38
,, ,,	−24·1	−32·2	2·39	9·70
,, ,,	−21·0	−35·5	3·28	15·2
,, sheep	−11·1	−26·7	0·60	3·60
,, ,,	−13·9	−27·1	1·77	7·76
,, ,,	−13·6	−21·7	0·93	4·39
,, guinea-pig	—	—	—	—
Liver, guinea-pig	− 7·86	− 8·10	—	—
,, sheep	− 3·11	− 3·87	—	—
Intestine (ileum), rat	−11·9	−13·0	2·36	2·30
,, (jejunum), rat	−10·5	−10·2	1·40	1·57
,, (duodenum), rat	−10·2	−11·9	1·29	1·54
,, (coecum), rat	− 7·02	− 7·18	0·57	0·81
,, (jejunum), guinea pig	− 7·05	− 6·95	0·20	0·43
,, (duodenum), guinea pig	− 6·86	− 6·59	0·41	0·54
Muscle, abdominal wall, rat	− 2·84	− 3·42	0·19	0·20
Kidney, pig	−13·5	−17·3	1·05	3·18
Liver, pig	− 3·85	− 2·7	0·68	0·64

production. In liver both changes are only slight. Other amino-acids show similar differences. Since however the amount of liver tissue in the body is (roughly) five times as much as the amount of kidney cortex, the share of the liver in deamination is considerable. Besides, the amino-acids coming from the intestinal tract are at a higher concentration when they reach the liver than when they reach the kidney. It may be therefore that the amino-acids of the food are preferentially deaminated in the liver.

Slight increases in oxygen uptake and ammonia formation are frequently observed if *l*-amino-acids are added to intestinal wall, especially the lower part (caecum) (see also London *et al.* [1934]), although the increase in ammonia formation is only about a tenth of that in kidney. Addition of amino-acid to rat diaphragm does not cause an increase in oxygen uptake or ammonia formation. Minced muscle however shows increased oxygen uptake in the presence of *l*-aspartic acid and *l*(+)glutamic acid [Needham, 1930]. The rate of oxidation in muscle is very slow and no ammonia is found if these amino-acids are present.

The mouse Crocker tumour and the following rat tissues do not show a measurable increase in ammonia formation if *l*-aspartic acid or *dl*-alanine is added to slices suspended in glucose-containing (0·2 %) saline; brain, retina, spleen, testicle, placenta, chorion, red blood corpuscles, red bone marrow, pancreas, salivary glands, Jensen rat sarcoma.

However, brain and retina have an increased respiration in the presence of glutamic acid. The respiration of brain or retina *in vitro* falls off quickly if no suitable substrate is added. Suitable substrates are carbohydrates (glucose, fructose), lactate and pyruvate. *l*(+)Glutamic acid is the only amino-acid which I have found capable of maintaining the respiration of brain or retina (Tables XXV and XXVI and Fig. 7). This is in accordance with Quastel and Wheatley's [1932] results. But the solution in which glutamic acid is oxidised does not contain an increased amount of ammonia. Glutamic acid seems to influence the respiration without being deaminated. This phenomenon will be dealt with fully in a subsequent paper.

Table XXV. *Influence of* l(+)*glutamic acid on brain respiration.*

Rabbit brain cortex in phosphate saline. 37·5°. O_2.

	Oxygen uptake per mg. dry tissue (μl.)			
Time (mins.)	No substrate added	*M*/50 *l*(+)glutamic acid	*M*/30 glucose	*M*/30 glucose *M*/50 *l*(+)glutamic acid
20	2·59	4·69	3·38	5·86
40	4·35	8·90	6·50	11·4
60 (=Q_{O_2})	5·62	12·9	9·48	16·8
80	6·62	16·5	12·4	21·6
100	7·37	19·8	15·3	26·0

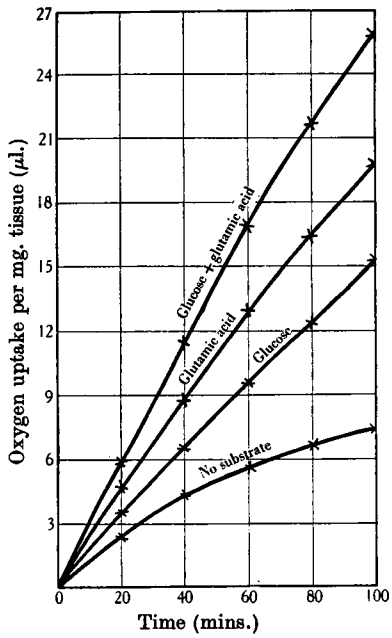

Fig. 7. Effect of *l*(+)glutamic acid on the respiration of rabbit brain.

Table XXVI. *Influence of* l(+)*glutamic acid on retina respiration.*

Pig retina in phosphate saline. 37·5°. O_2. Q_{O_2} is calculated for the first hour of the experiment. For (1) and (2) and for (3) and (4) the retinae of the same animal were used.

	(1)	(2)	(3)	(4)
	No substrate added	*M*/50 *l*(+)glutamic acid	*M*/30 glucose	*M*/30 glucose *M*/50 *l*(+)glutamic acid
Q_{O_2} (*a*)	−7·3	−13·9	− 9·65	−18·0
(*b*)	−3·8	−12·3	−10·4	−14·3

VI. Cell structure and enzyme action.

(1) *Deamination of* l-*amino-acids in tissue "brei". Effect of dilution.* The main difference between the *d*-amino-acid and *l*-amino-acid deaminases appears to be the behaviour on extraction of the ground tissue. The *d*-amino-acid deaminase can be extracted and the *l*-amino-acid deaminase cannot. This

difference is not merely a difference in solubility since, in the case of the *l*-amino-acid deaminase, both the supernatant fluid and the insoluble residue of the ground tissue are inactive.

The *l*-amino-acid deaminase belongs to the large category of cellular oxidations which are said to be bound up with the structure of the living cell. The bulk of the cell respiration also disappears when the tissue is ground and extracted.

In order to analyse the difference between the two deaminases, the process of extraction was studied in detail. Experiments described in this section show that the enzyme is inactivated, not by grinding and destruction of the cell, but by the dilution of the protoplasm which necessarily accompanies extraction. The extent of the dilution determines the extent of the inactivation. This is true for the *l*-amino-acid deaminase and for the bulk of the cell respiration.

Table XXVII shows some examples of the effect of grinding and dilution on *l*-amino-acid deaminase. For these experiments, kidneys of larger animals were obtained from the slaughter house as soon as possible after the death of the

Table XXVII. *Deamination of* l(−)*aspartic acid in kidney "brei".*

For details see text.

Tissue	Temperature ° C.	Final dilution of the tissue	Time (mins.)	Ammonia formed (μl.) Blank	With l(−)aspartic acid	With l(−)aspartic acid corrected for blank
Pig kidney	37·5	4-fold	50	82	330	248
,,	37·5	8-fold	50	55	67·5	12·5
Pig kidney*	37·5	4-fold	30	76·5	127	50·5
,,	37·5	8-fold	30	42	74	32
,,	37·5	16-fold	30	41·5	53	11·5
Pig kidney	37·5	4-fold	40	118	375	257
,,	37·5	8-fold	40	58	70	12
Sheep kidney	37·5	4-fold	40	159	242	83
,,	37·5	8-fold	40	108	125	17

* Kidney kept on ice for 3 hours before the experiment.

animal. The cortex was minced in a "Latapie" mincer. To the mashed tissue, one volume of phosphate-saline was added. The mixture was thoroughly shaken in a stoppered measuring cylinder. This suspension of the whole kidney was used for the experiments. It can be conveniently measured with a pipette.

The tissue in this suspension is diluted by one volume. This dilution is unavoidable since the concentrated mince is semi-solid and cannot be saturated with oxygen and with substrate. 0·5 ml. of the suspension (about 60 mg. dry weight) was measured into each Warburg flask and 0·5 ml. of *M*/5 l(−)aspartate was added. Varying amounts of phosphate-saline were added for further dilution. Thus the flasks contained a constant amount of tissue and amino-acid, but a varying amount of suspension fluid. Controls were carried out without l(−)aspartate. The gas space was filled with oxygen. The shaking was fast since the oxygen uptake was very large in the concentrated suspensions. In the "brei" that was diluted 4-fold, the rate of ammonia formation from l(−) aspartic acid is still of the same order of magnitude as it is in slices. With 8- or 16-fold dilution, the deamination is much inhibited, in some experiments almost completely.

(2) *Effect of dilution on respiration of tissue "brei".* Tables XXVIII and XXIX show the effect of dilution on the respiration of kidney "brei". The more

Table XXVIII. *Influence of dilution on the oxygen uptake of tissue "brei"*.*

	Tissue (wet weight)	Temperature ° C.	Dilution with saline	Oxygen uptake (μl.) in 5 mins.	10 mins.	20 mins.	40 mins.
1	Pig kidney (500 mg.)	19	2-fold	61	118	245	486
	,,	19	4-fold	38	80	138	245
	,,	19	8-fold	21	41	69	111
2	Pig kidney (250 mg.)	37·5	4-fold	49	71·5	126	—
	,,	37·5	8-fold	15	28	51	—
3	Sheep kidney (500 mg.)	20	2-fold	—	—	158	320
	,,	20	6-fold	—	—	53	110
4	Sheep kidney (500 mg.)	37·5	2-fold	54	113	196	—
	,,	37·5	6-fold	30	68	93	—
5	Sheep kidney (500 mg.)	37·5	2-fold	115	232	425	—
	,,	37·5	4-fold	60	117	187	—
6	Sheep kidney (500 mg.)	37·5	2-fold	121	238	441	—
	,,	37·5	6-fold	38	79	141	—

* Addition of glucose or *l*(−)aspartic acid to diluted "brei" had no effect on the oxygen uptake.

Table XXIX. *Oxygen uptake in slices and in "brei".**

Tissue	Temperature ° C.	Q_{O_2} in slices	Q_{O_2} in "brei"* diluted 2-fold	4-fold	8-fold
Pig kidney	19	− 2·77	− 7·22	− 3·17	− 1·73
Pig kidney	20	− 3·83	− 4·67	—	—
Sheep kidney	20	− 3·65	− 4·15	—	—
Sheep kidney	37·5	− 15·5	− 12·0	− 6·05	—

* The dry weight of kidney cortex was found to be 23 % of the wet weight.

concentrated suspensions respire about as much as slices, sometimes even more. Dilution destroys the respiration more or less in proportion to the extent of the dilution.

Macfadyen *et al.* (for references see Harden [1932]) and Warburg [1911; 1914] have described similar observations. Macfadyen *et al.* found that the alcoholic fermentation of yeast juice disappears with dilution. Warburg investigated the effect of dilution on the respiration of laked blood corpuscles of the goose. He cytolysed the cells by freezing and thawing. The oxygen uptake of the concentrated cytolised red cells was 60–75 % higher than that of the intact cells. When the cells were diluted in the proportions 2:5:11, the oxygen consumption was in the proportions 66:31:15. Thus the increase of respiration after destruction of the cells and the decrease on dilution are very similar in red cells and in kidney.

It should be mentioned that the experiments described in this section were carried out with freshly prepared "brei". "Brei" which had been kept on ice for 24 hours gave only a small oxygen uptake as compared with fresh "brei".

These results lead to a distinction between enzymes or systems which act independently of the amount of fluid in which they are dissolved or suspended and systems which act only within a small range of concentration and are destroyed if the medium is diluted. The vast majority of the common enzymes belong to the first group. The second group comprises those reactions which have generally been considered as being bound up with the structure of the living cell, *i.e.* the bulk of oxidations and fermentations.

(3) *Theory of the effect of dilution.* The effect of dilution can be explained by the assumption that a reaction between more than two partners (a ternary collision or a collision of a higher order) determines the velocity of the reaction. Such components may be for instance activated substrate, activated oxygen and a co-enzyme. The probability of a ternary collision decreases in proportion to the dilution. If one volume of enzyme solution is diluted with one volume, the number of ternary collisions is halved in the total volume of the mixture. In many experiments in Table XXVIII, the effect of dilution is that expected for a ternary collision.

Ternary collisions occur extremely rarely in homogeneous solutions. It is one of the functions of the structure of the cell to arrange the catalysts in such a way that a ternary collision reaches a certain degree of probability. Of course this is not the only reason why the structure is of importance for chemical reactions in cells. It is not however within the scope of this paper to discuss this problem in full.

Thus the different behaviours of the *d*- and *l*-amino-acid deaminases on extraction and dilution appear to be due to the different structures of the two enzymic systems. The *d*-enzyme is a comparatively simple system, the *l*-enzyme a more complicated one in which an additional factor, involving a ternary collision, plays a part.

VII. Interrelation between *d*- and *l*-amino-acid deaminases.

From the experiments described, it was concluded that two different deaminating systems exist. But it must be pointed out that this difference only concerns the two systems as whole systems. It may well be that the two systems have certain components in common; for instance it may be that the *l*-deaminase is the *d*-deaminase *plus* an additional factor. The idea that the *d*-deaminase is a fragment of the *l*-amino-acid deaminase is supported by the fact that *d*-deaminase can be obtained only from those tissues which contain the *l*-system, and by the fact that the *l*-system cannot be separated from the *d*-system. Moreover it would explain the occurrence of an enzyme for which practically no substrate is found in nature. If this view is correct, the additional factor, which makes the *d*-enzyme into the *l*-enzyme, is a substance which reacts with *l*-amino-acids and enables them to react in the same way as *d*-amino-acids react by themselves without an auxiliary substance.

Summary.

1. Slices of liver and kidney deaminate (by oxidation) the optically "natural" amino-acids and also their optical isomerides which do not occur naturally.

2. The "natural" amino-acids, which according to their spatial configuration belong to the *l*-series, are not deaminated in the presence of octyl alcohol or *M*/100 cyanide, or in extracts of ground tissue or dried tissues.

3. The "non-natural" amino-acids which belong to the *d*-series are deaminated in the presence of octyl alcohol, of *M*/100 cyanide and in extracts of ground or dried tissues.

4. These differences between the deaminations of optical isomerides are explained by the assumption that deamination of the two stereoisomerides is brought about by two different enzymic systems ("*d*-amino-acid deaminase" and "*l*-amino-acid deaminase"). The difference concerns the two systems as whole systems; it may be that they have certain components in common or even that the *d*-amino-acid deaminase is a fragment of the *l*-amino-acid deaminase.

5. The activity of the deaminating systems under various conditions and their occurrence in various tissues and animals have been investigated.

6. The *l*-amino-acid deaminase is active in ground tissue when the "brei" is suspended in a small volume of fluid. On dilution of the tissue suspension, the activity disappears approximately in proportion to the dilution.

7. The cell respiration in ground tissue shows a similar sensitivity towards dilution.

8. The effect of dilution is explained by the assumption that a ternary collision, or a collision of a higher order, plays a role in those enzymic systems which become inactive on dilution. The probability of a ternary collision decreases in proportion to the dilution.

9. The deamination of *l*- and *d*-amino-acids in kidney slices is inhibited by substances which can be oxidised by kidney. If two different substances, each of which causes an increase in oxygen consumption, are added together to kidney or yeast, no summation of the effects on the oxygen uptake ensues. The substrates compete for the oxygen and displace each other. This indicates that the process of activation of oxygen is identical for all the substrates which show no summation.

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REFERENCES.

Abderhalden and Tetzner (1935). *Z. physiol. Chem.* **232**, 79.
—— and Kautsch (1910). *Z. physiol. Chem.* **68**, 487.
Alt (1930). *Biochem. Z.* **221**, 498.
Banga, Schneider and Szent Györgyi (1931). *Biochem. Z.* **240**, 462.
Bergmann and Köster (1926). *Z. physiol. Chem.* **159**, 179.
Bernheim and Bernheim (1934). *J. Biol. Chem.* **106**, 79.
—— —— (1935). *J. Biol. Chem.* **109**, 31.
Bornstein and Budelmann (1930). *Biochem. Z.* **218**, 64.
Clarke and Inouye (1931). *J. Biol. Chem.* **94**, 548.
Cremer (1929). *Biochem. Z.* **206**, 228.
Dickens and Greville (1933). *Biochem. J.* **27**, 1123.
Dixon and Elliott (1930). *Biochem. J.* **24**, 820.
Ehrlich (1906). *Biochem. Z.* **1**, 8.
—— (1914). *Biochem. Z.* **63**, 397.
—— (1927). *Biochem. Z.* **182**, 245.
Fieser (1924). *J. Amer. Chem. Soc.* **46**, 2639.
Fischer, E. (1908). *Ber. deutsch. chem. Ges.* **41**, 893.
Fischer, H. O. L. and Feldmann (1932). *Ber. deutsch. chem. Ges.* **65**, 1211.
Fränkel and Gallia (1923). *Biochem. Z.* **134**, 308.
—— —— Leibster and Rosen (1924). *Biochem. Z.* **145**, 225.
Harden (1932). Alcoholic fermentation. (Longmans, Green and Co., London.)
Harington and Randall (1931). *Biochem. J.* **25**, 1917.
Hasselbalch (1915). *Biochem. Z.* **74**, 18.
Knoop (1925). *Z. physiol. Chem.* **148**, 294.

Krebs (1933, 1). *Z. physiol. Chem.* **217**, 191.
—— (1933, 2). *Z. physiol. Chem.* **218**, 157.
v. Lippmann (1884). *Ber. deutsch. chem. Ges.* **17**, 2835.
London, Dubinsky, Wassilewskaja and Prochorowa (1934). *Z. physiol. Chem.* **227**, 223.
Nash and Benedict (1921). *J. Biol. Chem.* **48**, 463.
Needham (1930). *Biochem. J.* **24**, 208.
Neubauer (1909). *Deutsch. Arch. klin. Med.* **95**, 211.
—— (1928). Handbuch der normalen und pathologischen Physiologie, **5**, 775.
Parnas (1934). *Biochem. Z.* **274**, 158.
—— and Heller (1924). *Biochem. Z.* **152**, 1.
—— and Klisiecki (1926). *Biochem. Z.* **173**, 224.
Patey and Holmes (1930). *Biochem. J.* **24**, 1564.
Quastel and Wheatley (1932). *Biochem. J.* **26**, 725.
Thunberg (1920). *Skand. Arch. Physiol.* **40**, 58.
—— (1923). *Skand. Arch. Physiol.* **43**, 275.
Van Slyke and Meyer (1912). *J. Biol. Chem.* **12**, 399.
—— —— (1913). *J. Biol. Chem.* **16**, 187, 197, 213, 231.
du Vigneaud and Sealock (1932, 1). *J. Biol. Chem.* **96**, 511.
—— —— and van Etten (1932, 2). *J. Biol. Chem.* **98**, 565.
Walker (1889). *Z. physikal. Chem.* **4**, 319.
Warburg (1911). *Z. physiol. Chem.* **70**, 413.
—— (1914). *Ergebn. Physiol.* **14**, 323.
—— (1926). Stoffwechsel der Tumoren. (Springer, Berlin.)
—— (1931). *Biochem. Z.* **231**, 493.
—— and Christian (1931). *Biochem. Z.* **242**, 206.
—— —— (1932). *Biochem. Z.* **254**, 438.
—— and Onaka (1911). *Z. physiol. Chem.* **70**, 433.
Wohl and Freudenberg (1923). *Ber. deutsch. chem. Ges.* **56**, 309.