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A Method for the Assay of Insulin by Paper Chromatography

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Methods for the assay of insulin *in vitro* have appeared at intervals in the literature. Among the most familiar are the fibril-precipitation method of Waugh, Thompson & Weimer (1950) and the more recent version of the same method (Grodsky, 1958). The former method was the subject of an investigation by Foster, Macdonald & Smart (1951), who, although able to obtain reliable results in assays on freshly prepared solutions of insulin, protamine insulin and commercial concentrates, reported failure with solutions of low-potency insulin (11 units/mg.), with globin insulin and with a 5½-year-old solution of insulin.

Numerous chromatographic methods for the separation of insulin from associated impurities may be found in the literature. Porter (1953) separated insulin from crude preparations by using a silane-treated kieselguhr column. Porath & Li (1954) used a charcoal column successfully and Dickinson (1956) a column of calcium phosphate and kieselguhr.

Other workers have achieved satisfactory separation of insulin from other proteins by paper

chromatography; Robinson & Fehr (1952) were able to estimate the percentage of insulin in protamine zinc insulin, and Grodsky & Tarver (1956) separated insulin from liver proteins and pancreatic extracts. Light & Simpson (1956) achieved separation from similar mixtures.

The high cost of biological assays of insulin has always suggested that a more economical process would be of great value, and it appeared worthwhile investigating the possibility of adapting a chromatographic method to fulfil this need.

There is abundant evidence in the literature for the heterogeneity of insulin samples, and it seemed reasonable to assume that a suitable solvent system might be capable of separating biologically active insulin from the impurities with which it is associated. If this could be done, there should be a relationship between the amount of biologically active insulin separated from a given amount of sample and the biological activity of the sample on a weight basis.

Light & Simpson (1956) found the biological activity of insulin to be associated with a specific

spot on their chromatograms, and with their solvent system it has been found that the amount of dye bound to this specific spot by the staining technique to be described bears a linear relationship to the content of biologically active insulin in the sample of origin.

This relationship constitutes the basis of the method to be described.

EXPERIMENTAL

Chromatography tanks. Rectangular glass tanks (height 42 cm., base 27 cm. $\times$ 9 cm.) with ground tops and $\frac{1}{8}$ in. plain glass lids sealed on with silicone grease were used. Each lid was drilled centrally to accommodate a rubber stopper through which passed a short length of 7 mm. diam. glass tube, serving to guide the vertical member of an inverted T-piece of glass rod down into the tank. The horizontal member of the T-piece formed a support rail within the tank, from which the chromatograms could be suspended. This assembly was designed to allow adjustment of the position of the chromatograms within the sealed tank by external manipulation of the protruding vertical rod. A small circle of stiff sheet rubber with a hole made in its centre was pushed down over the protruding end of the vertical rod to retain the rod in the desired position.

Volumetric vessels and dispensers. In order that the assay may be run on not more than 10 mg. amounts each of sample and standard, a semi-micro technique was developed for preparation of the insulin solutions. The method required the use of volumetric vessels of approx. 1 ml. capacity, which were unobtainable from commercial sources and were therefore made in the Laboratory from glass tubing of 8 mm. diam. by sealing one end and forming a constricted neck (approx. 2 mm. diam.), 5 cm. from the sealed end, and making a graduation mark with a diamond pencil in the centre of the neck. The vessels were then calibrated by filling to the graduation mark with water from a capillary pipette, and weighing on an analytical balance.

The precision of reproducibility was tested by performing four weighings on each of nine vessels made and calibrated in this way. The mean deviations were max. 0.180% and min. 0.0495%. The volumes of the vessels tested ranged from 1.1824 to 1.4016 ml.

The semi-micro dispensers of Stock & Fill (1956), made with glass tubing of 2.5 mm. bore and suitably fitting glass rod, were found convenient for introducing the crystalline insulin samples into the vessels for weighing.

Developing solvent. Butan-2-ol-1% acetic acid (1:1, v/v) (Light & Simpson, 1956) was shaken vigorously for several minutes in a large separating funnel and placed in the chromatography room for 24 hr., after which the lower phase was discarded and the upper butanol phase run into the bottom of the chromatography tanks to give a layer of 1.0-1.5 cm. depth. Laboratory Reagent-grade butan-2-ol and AnalaR acetic acid (British Drug Houses Ltd.) were used in the preparation of the developing solvent.

Protein stain. Acetic acid (12 ml.) was diluted to 2 l. and 125 ml. of 0.1N-NaOH added. Bromocresol green was dissolved in this solution to 0.02% (w/v). The final pH was 3.6. The stain was renewed at approximately fortnightly intervals when in daily use.

Eluent. 0.1N-NaOH-95% ethanol (1:1, v/v) was used. The ethanol content of this eluent prevented disintegration of the paper when eluting the dye, thus obviating the necessity for centrifuging or filtration of the eluates before colorimetric measurement.

The linearity of the colorimetric measurement of the dye eluted from the chromatograms was checked by applying spots (10 μ l.) of solution of crystalline insulin in 0.05N-HCl to Whatman no. 1 paper to give a range of 0-110 μ g. of sample. The chromatograms were developed, stained and eluted by the method described below. Extinctions of the eluates at 625 m μ were plotted against concentration of sample (Fig. 1). A linear relationship between the weight of sample applied and the extinctions of the eluates was found over the entire range.

For working purposes, the middle portion of the range was chosen, the concentration of solutions for assay being so arranged that 40-60 μ g. of sample could be applied to the papers in a convenient and reproducible volume.

Assay method

With the semi-micro dispenser, approx. 10 mg. amounts of test sample and of insulin standard are weighed into calibrated volumetric vessels on an analytical balance. The vessels are placed in a desiccator over P₂O₅, their contents dried *in vacuo* to constant weight (24-48 hr.) and moisture contents recorded.

0.05N-HCl is added with a capillary pipette to the vessels, which are whirled between the palms of the hands for 1 min. to dissolve the contents; more acid is added, to the calibration mark, and the contents of the vessels are thoroughly mixed by repeatedly drawing into and expelling from dry capillary pipettes fitted with rubber teats.

Two spots of test solution, and two of insulin standard, are applied to each of 12 sheets of Whatman no. 1 paper (28.5 cm. long $\times$ 13 cm. wide) at intervals of 2.5 cm., upon a line drawn 4 cm. from the bottom edge of the paper. The applications are made with an Agla micrometer syringe and size S.S. Record needle, the assembly being mounted vertically with the needle point in light contact with the surface of the paper. A thin film of petroleum jelly applied to the needle near the point prevents the solution from wetting the outside of the needle.

Fig. 1. Effect of difference in biological potency of two insulin samples on the slope of extinctions of eluted spots/weight of sample chromatographed. ●, 0-110 μ g. (dry wt.) of insulin at 22.95 units/mg.; ○, 0-110 μ g. (dry wt.) of insulin at 22.30 units/mg.

The precise volume to be expelled from the micrometer syringe is calculated from the weight of sample and the capacity of the volumetric vessel in which the solution was prepared, and should be such that 40–60 $\mu\text{g.}$ of sample is deposited per spot. The diameter of each spot should not exceed 1 cm. After drying at room temperature for 2–24 hr., a fold is made 2.5 cm. from the top edge of each paper and a hole punched centrally through the flap and paper with a paper punch. The papers are then assembled in the tanks, four or five papers per tank, but with the rods retracted so that the papers are out of contact with the solvent; the folded tops of the papers form a series of corrugations, serving to separate the papers from each other. After equilibration for 4–16 hr. in this position, the rods are pushed downwards until 2–4 mm. of the bottom edge of each paper becomes immersed in the solvent; development is allowed to proceed until the solvent front has travelled at least 20 cm. up the paper. It has been found convenient to equilibrate overnight, then to allow development to proceed during the whole of the next day.

Chromatography is carried out at room temperature in a room reasonably free from draughts. It has not been found necessary, however, to apply any form of temperature control to the room.

The papers are removed from the tanks, pressed between sheets of clean blotting paper to remove excess of solvent and dried in an electric air oven at 80° for 10 min. They are then immersed in the protein stain for 15 hr. (overnight), washed four times for 3 min. in 1% (v/v) acetic acid with occasional agitation, then pressed between sheets of clean blotting paper and returned to the air oven for 10 min. to dry. The papers are then held over a beaker containing aq. 5N-NH₃ soln. to intensify the dye colour, and the specific insulin spots (those with the highest R_F) are circumscribed with pencil, cut out and placed in a series of 12 cm. $\times$ 1.5 cm. test tubes. A blank, of size similar to the spots, is cut from a portion of the paper remote from the spots but within the solvent areas, and placed in a separate tube. To each tube is added 5 ml. of eluent, and the tubes are agitated at intervals during 30 min.; then the extinctions are compared with that of the blank eluate, in a Unicam SP. 600 spectrophotometer at 625 m μ with 10 mm. cells.

No colour instability has been observed for periods of up to 10 days, but precautions should be taken to avoid evaporation of the eluent if the colours cannot be read at once.

The assay result of the test sample is calculated from the following expression:

$$\text{Activity of test sample (units/mg.)} = \frac{W_s \times V_s \times C_t \times \bar{M} \times U}{W_t \times V_t \times C_s},$$

where W_s is weight of standard (mg.) before drying; V_s is volume of standard (ml.) applied per spot; C_t is capacity (ml.) of volumetric vessel containing test sample; U is units/mg. of standard (not corrected for moisture content); W_t is weight of test sample (mg.) before drying; V_t is volume (ml.) of test solution applied per spot; C_s is capacity (ml.) of volumetric vessel containing standard; $\bar{M}$ is the geometric mean of the ratios of extinctions of test samples/extinctions of standards.

$\bar{M}$ is substituted into the above expression after its value has been calculated from the following equation:

$$\bar{M} = N \sqrt[\frac{1}{N}]{\left(\frac{X_t}{X_s}\right)_1 \cdot \left(\frac{X_t}{X_s}\right)_2 \dots \left(\frac{X_t}{X_s}\right)_N}$$

in which X_t is the extinction of the eluate from an inner or outer spot of test sample; X_s is the extinction of the eluate from the corresponding inner or outer spot of standard from the same paper; N is the number of observations.

For convenience of working, the expression is converted into logarithms to base 10, when

$$\bar{M} = \text{antilog} \left[\log \left(\frac{X_t}{X_s} \right)_1 + \log \left(\frac{X_t}{X_s} \right)_2 + \dots + \log \left(\frac{X_t}{X_s} \right)_N \right] \frac{1}{N}.$$

If for any reason (breakages, etc.) a test observation were lost, the corresponding standard observation from the same paper would be omitted.

The values W_s and W_t are given in terms of insulin samples 'uncorrected for moisture content', since it is customary to express insulin potencies on this basis, and to state the moisture contents with the assay results.

RESULTS

The method was tested by assaying a series of samples of known biological potency, ranging from crystalline zinc insulin of high activity through amorphous insulins having intermediate activities to crude hydrochlorides of low value. Results are shown in Table 1 and in Fig. 2.

There were also available several vials of insulin solution (40 units/ml.; insulin injection, B.P.), which had been stored for a prolonged period of time under unfavourable conditions. The contents of these vials were divided, one half being assayed by the biological-control laboratories by the mouse-convulsion test (Young & Stewart, 1952) and the other half assayed in this Laboratory by the chromatographic method.

These samples contained an accumulation of clear thixotropic gel as a result of their prolonged storage. This gel is the inactive form of insulin referred to by Foster *et al.* (1951), and was removed by centrifuging at 2000 rev./min. in a MSE Super Minor centrifuge for 15 min. before chromatography, since the viscosity of the samples would have impaired the accuracy with which the measured volumes could be applied to the papers. The validity of this procedure is dealt with in the Discussion. Results are shown in Table 2.

In order to obtain a statistical assessment of the errors inherent in the chromatographic part of the method, an experiment was carried out with a balanced factorial design covering the following recognizable sources of error: (i) errors 'within papers', particularly with respect to inner and outer positions on the papers; (ii) errors between papers, within tanks; (iii) errors between tanks; (iv) errors between days.

Although the last item cannot reliably be estimated in a single experiment because of the possible influence of atmospheric factors, it was included in the design to provide information on the effect of storing prepared papers. It was decided therefore

Fig. 2. Insulin chromatograms showing different colour intensities of developed spots (insulin-dye complex), with insulin samples of different potencies. 55 μ g. of each sample, and 55 μ g. of standard, were applied in duplicate to each paper. Chromatograms were developed for 15 hr. A, 0.5 unit/mg.; B, 9.0 units/mg.; C, 18.0 units/mg.; D, 23.0 units/mg.; S, standard, 23.04 units/mg.

Table 1. *Comparison of biological and chromatographic assay on crystalline and amorphous insulin samples*

No.	Description of sample	Biological assay		Chromatographic assay	
		(Units/mg.)	(Limits, $P=0.95$)	(Units/mg.)	(Range)*
140	Crystalline insulin	20.5	(20.5-23.8)	21.5	(20.3-23.2)
146	Crystalline insulin	23.3	(21.7-25.2)	23.1	(21.6-24.7)
B.D.H. P/5	Crystalline insulin	23.04	—	22.8	(22.0-23.2)
AN 67224	Crystalline insulin	21.9	(19.9-24.2)	21.5	(20.15-22.39)
365/24/A3	Crystalline insulin	25.1	($\pm 20\%$ approx.)	23.6	(22.4-24.8)
365/25/A3	Crystalline insulin	18.0	($\pm 20\%$ approx.)	18.1	(17.5-18.7)
365/97	Amorphous insulin	18.0	(15.7-20.5)	17.7	(16.8-18.7)
365/113/B	Amorphous insulin	15.0	($\pm 20\%$ approx.)	15.6	—
365/113/A	Amorphous insulin	13.0	($\pm 20\%$ approx.)	15.06	(14.9-15.2)
347/97/A2	Crude insulin hydrochloride	0.567	($\pm 100\%$ approx.)	1.04	—
347/98/A2	Crude insulin	0.438	($\pm 100\%$ approx.)	1.46	(1.18-1.88)

* These assays were performed against a standard of 22.3 units/mg., which had been specially assayed by the mouse-convulsion method between limits ($P=0.95$) of 20.6 and 24.1.

Table 2. *Comparison of biological and chromatographic assays on samples of 'insulin injection', after prolonged storage under unfavourable conditions*

The samples contained 40 international units/ml. when originally prepared, and had decayed as a result of storage, leaving the residual potencies shown in the table.

Sample	Biological assay		Chromatographic assay	
	(Units/ml.)	(Limits, $P=0.95$)	(Units/ml.)	(Range)*
4704 A	26.4	(22.4-30.3)	27.2	(26.7-27.7)
4712 A	24.5	(20.6-27.9)	27.35	(26.4-28.3)
4792 A	26.2	(23.3-29.5)	23.7	—

* These assays were performed against a standard of 22.3 units/mg., which had been specially assayed by the mouse-convulsion method between limits ($P=0.95$) of 20.6 and 24.1.

to run the trial on four occasions, each 3 days apart, the papers having all been prepared on the first day. On each day, 16 papers were evenly distributed in a designated order of position between four tanks. Each paper carried two spots of standard insulin solution and two spots of test solution placed so as to cover all possible positions on the papers, and in particular to ensure that each sample and each standard occupied inner and outer positions on the papers.

The analysis did not allow for contributions to error involved in preparation of the solutions for testing, since the design of the experiment required that samples of the same pair of solutions be applied to each paper.

A full factorial analysis of variance was carried out on the completed set of spectrophotometer readings, which, since they may be regarded as proportional to the quantities of sample applied to the spots, were converted into log units for analysis; twenty-one of the interactions were not significant against the residual, and since these twenty-two variances were homogeneous, they were combined to provide a better estimate of the overall error. The shortened analysis is given in Table 3.

The conclusions to be drawn from experiments designed to provide statistical evidence may be summarized as follows: (i) there is a significant difference between the standard insulin and the test sample, the order of which is not affected by the site or time of testing, provided that the samples of test and standard are set up in parallel situations at the same time; (ii) the inner and outer positions on the papers give substantially similar

results; (iii) a difference is observed between papers within tanks. Since the comparison of tanks is confounded with differences due to papers it is probable that the tanks themselves do not contribute appreciably to the error; (iv) the lowering of readings over the four test occasions is most significant (see Table 3). The high first- and second-order interactions of papers and tanks with days suggests that the deterioration is not uniform for all papers. The 'within-paper' error might therefore increase with prolonged storage.

A separate analysis of the results for each day of the test (Table 4) indicates that a prolonged period of storage between preparation of the papers and chromatography is highly undesirable.

The error variance for the first day (0.00017 in log units) may be taken as a reasonable guide to the inherent accuracy of the method. Since it refers to a single colorimetric determination, the variance of a relative potency of one sample in terms of another will be the variance of the difference of their log values, i.e. 0.00034 (Table 4). The log ratio of potency of a single observation, each on standard and test, would therefore have a standard deviation of $\sqrt{0.00034}$, or ± 0.0184 , corresponding to $\pm 4.3\%$.

The 'statistical weight' of an assay is the reciprocal of the variance. Therefore an assay involving a single determination on each of standard and test would have an estimated weight of $1/0.00034$, i.e. approx. 2900. This weight is about ten times as great as the weight for the assay of insulin by the mouse-convulsion method [Young & Stewart (1952); Smith (1950)] with a total of 96 mice in a 2×2 design.

Table 3. *Analysis of variance (abridged table)*

Source of variation	Degrees of freedom	Mean square	P
Between samples	1	0.03224	<0.001
Between positions on paper	1	0.00101	0.1-0.2
Between papers within tanks	3	0.00259	0.01-0.001
Between tanks	3	0.00432	<0.001
Between days: linear	1	1.06070	<0.001
quadratic	1	0.00143	0.1
cubic	1	0.03871	<0.001
Papers $\times$ tanks	9	0.00340	<0.001
Papers $\times$ days	9	0.00790	<0.001
Tanks $\times$ days	9	0.02024	<0.001
Papers $\times$ tanks $\times$ days	27	0.00615	<0.001
Error	190	0.00052	—

Table 4. *Analysis for samples and papers on four test occasions*

Source of variance	Degrees of freedom	Mean squares			
		First day	Second day	Third day	Fourth day
Between samples	1	0.01479	0.00799	0.00797	0.00347
Between papers	15	0.00156	0.00470	0.00532	0.01979
Error	47	0.00017	0.00040	0.00079	0.00071

From the data obtained on the four test days, the weighted mean ratio of test to standard was 1.06, with a statistical 'weight' of 176 907. The overall mean for the entire experiment indicates that the test material was 1.06 times as rich in biologically active insulin as the standard. The very great weight associated with this estimate corresponds to limits of error of slightly over $\pm 1\%$ ($P=0.95$), which, of course, does not allow for errors in making up solutions.

From the statistical analysis, three recommendations may be made: (i) when calculating results, test samples should be compared with individual standards applied in similar positions (inner or outer) on the same paper; (ii) papers should not be stored longer than necessary between application of the samples and chromatography; (iii) extra replication for increased accuracy should be limited to increasing the number of solutions made up, rather than merely increasing the number of papers prepared from each solution.

DISCUSSION

In the early part of the experimental work, comparisons were made between solvent systems containing butan-1-ol and butan-2-ol, each with acetic acid with water. It was found that whereas insulin could satisfactorily be estimated on a weight basis with the former solvent, by the method of Robinson & Fehr (1952), it was only possible to relate the amount of insulin in the developed spot to the known biological activity of a sample where fairly pure insulin was used. The butan-2-ol system, however, was found to be superior in its ability to separate biologically active insulin from the impurities with which it is associated in the more crude samples examined, as is shown by the close agreement obtained between the biological-assay method and the chromatographic method.

Comparisons between ascending and descending chromatography showed the former to be more suitable for quantitative work, since the developed spots were generally more compact, and the compactness was found to be preserved during staining by using the protein stain as described.

Experiments on the concentration of acetic acid used in the solvent system showed that there was a positive relationship between the concentration of acid and the mobility of the insulin spot. However, any improvement in resolution due to increased mobility was outweighed by the disadvantage of increased temperature sensitivity of the more acid solvent; 'dephasing' was prone to occur in the tanks, consequent upon any slight increase in room temperature. There was therefore no advantage in varying the solvent from the original of Light & Simpson (1956), whose observation that

the R_F of insulin in this solvent varies with the amount of sample applied to the paper, and the distance travelled by the solvent front, has been confirmed.

When impure insulin samples are chromatographed with this solvent, many of the impurities which separate do not take up bromocresol green under the stated conditions, and therefore do not interfere with the assay. Their presence can be detected by spraying the papers with a 1% (w/v) solution of ninhydrin in acetone and heating them for 5 min. at 100° in an air oven.

Insulin solutions, after partial decay resulting from prolonged storage under unfavourable conditions, often contain, in addition to ninhydrin-positive substances, an accumulation of thixotropic gel, formed slowly as a result of changes occurring in solution during storage (Foster *et al.* 1951). In extreme cases, most of the insulin may be precipitated in this inactive form. If the gel be allowed to remain in the sample it remains fixed at the point of origin, along with other impurities, in which position it cannot interfere with the assay. Samples of gel isolated by centrifuging were freed from soluble insulin by washing repeatedly with water on the centrifuge (MSE Super Minor; 2000 rev./min., 10 min.), then chromatographed under the same conditions as an insulin sample. The protein stain revealed a very faint spot at the point of origin, apart from which the papers exhibited no further staining.

The results obtained by chromatography support the initial premise of the existence of a linear relationship between the biological activity of insulin appearing in the specific spot. The close agreement obtained between the mouse-convulsion test and the chromatographic method suggests that the specific spots contain insulin free from other material.

After the work described in this paper had been completed, we were privileged to receive the manuscript of a paper by J. Bouman and J. D. H. Homan, of the research laboratories of N.V. Organon, Oss, Holland. Their findings are similar to our own, but differences exist in the details of technique and in the method of calculation of results for the insulin assays.

SUMMARY

1. Insulin can be separated from associated impurities and inactive material by ascending chromatography with the upper phase of butan-2-ol, 1% acetic acid (1:1, v/v). A solution of bromocresol green buffered with sodium acetate is used for locating the insulin zones.

2. A linear relationship exists between the amount of insulin appearing in the specific zone and the biological potency of the sample.

3. By elution of the dye from the specific insulin spot and quantitative colorimetric comparison with the similar zone from a standard of known potency, the biological potency of the sample can be estimated.

4. Data are recorded on the comparison between biological potency and the potency given by the chromatographic method for samples of crystalline and crude insulins and for solutions of insulin which had undergone partial decay as a result of prolonged storage under unfavourable conditions. Close agreement has been obtained between the mouse-convulsion method and the chromatographic assay. The potency ratio of a single observation, each on test and standard, was found by statistical analysis to have a standard deviation corresponding to $\pm 4.3\%$.

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Protein Breakdown in *Bacillus cereus*

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In several recent studies, the proteins of *Escherichia coli* have been shown to be stable during growth. Thus Cowie, Bolton & Sands (1950) found that when cells which had been labelled with ^{35}S were grown further in unlabelled medium there was no release of labelled sulphur. Koch & Levy (1955), using a different technique for the detection of protein breakdown, concluded that only a small fraction (about 2%) of the total protein of *E. coli* breaks down during the first 2 hr. of growth, and that after this there is no degradation of protein. Again, it has been shown that during the synthesis of the inducible enzyme β -galactosidase, there is no utilization of material from the preformed proteins of the cells (Rotman & Spiegelman, 1954; Hogness, Cohn & Monod, 1955). These observations led Hogness *et al.* (1955) to suggest that not only bacterial proteins were metabolically stable, but that a similar conclusion might apply to the intracellular proteins of mammalian cells.

However, other investigations have been reported which do not support the general conclusion of Hogness *et al.* (1955). Evidence for intracellular-protein degradation in *E. coli* has been presented by Mandelstam (1957, 1958), who showed that whereas the proteins of rapidly growing cells appeared to be stable, those of non-growing cells were broken down to amino acids at the fairly high rate of 5%/hr. A similar value has been reported by Borek, Ponticorvo & Rittenberg (1958). Also, Halvorson (1958*a, b*), working with yeast, has found that protein breakdown in growing cells is only 4% of that in resting cells. Studies with mammalian systems have been carried out by Steinberg & Vaughan (1956) and Steinberg, Vaughan & Anfinsen (1956), who measured the release of labelled phenylalanine, alanine and leucine from rat-kidney and liver preparations, and its inhibition by dinitrophenol, amino acid analogues and during aerobic metabolism. Recently protein turnover in non-multiplying animal cells has been reported by Harris & Watts (1958), who have measured the incorporation and release of [^{14}C]valine in macrophages.

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