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ON THE APPLICATION OF AFFINITY CHROMATOGRAPHY TO TURNOVER STUDIES ON THE LACTATE DEHYDROGENASE ISOENZYMES

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Summary

1. The suitability of a combined application of the techniques of affinity chromatography, double labelling and gel electrophoresis in the determination of the turnover characteristics of the lactate dehydrogenase isoenzymes (L-lactate:NAD^+ oxidoreductase EC 1.1.1.27) in rat tissues has been studied.

2. Affinity chromatography was established as affording the advantages of rapidity, high yield and purity to such studies, and the double-labelling procedure was modified to encompass the differential decay kinetics in the separate rat tissues. In addition, a convenient method for the resolution and separate collection of radioactively labelled isoenzymes has been described.

3. Using this methodology, comparative turnover values for the isoenzymes of lactate dehydrogenase and for total soluble protein have been determined.

4. The comparability of these results with other methodologies, and the advantages of this approach in facilitating broad comparative studies on turnover are discussed.

Introduction

In recent years, there has been a marked increase in the emphasis placed on degradation as one of the major factors determining the levels of specific proteins in animal tissues [1], but, in general, a broad approach to turnover studies has been constrained by the demanding technical requirements of such investigations. Multiple isolations of an individual protein in a state of high purity is a common feature of such studies, for example, and in the case of multiple enzyme forms, these requirements are further compounded by the degree of multiplicity and structural inter-relationship in these systems.

In seeking a more apposite approach to turnover studies, and one which would allow ready application across a broad cross-section of phylogeny, the

powerful advantages of affinity chromatography were noted. Many specific proteins have now been isolated from complex mixtures, rapidly and in a high state of purity, by such procedures [2]. Additionally, it was recognized that the problem of multiple sampling might be overcome by the use of the elegant double-labelling technique of Arias et al. [3], in which the animal is separately injected with ^{3}H- and ^{14}C-labelled leucine over a suitable time interval, and the ratio of these labels in a particular protein used to determine the half-life [4].

In this paper, then, the possibility of combining the advantages of affinity chromatography and double-label techniques with the high degree of resolution afforded by gel electrophoresis, in furthering the study of the turnover characteristics of the lactate dehydrogenase isoenzymes has been investigated and substantiated.

Materials and Methods

Mature female rats of the local Wistar strain, and an average weight of 170 g, were used for all experimental work.

L-[4,5-^{3}H]Leucine (50—60 Ci/mmol) and L-[^{14}C]leucine (312—316 Ci/mol), both in 0.01 M HCl, were obtained from Schwarz - Mann Radiochemical Division, Orangeburg, New York.

1,6-Diaminohexane was supplied by B.D.H. Chemicals Ltd, Poole, England, and the potassium oxalate and cyanogen bromide by Ajax Chemicals Ltd, Sydney, Australia. Carbodiimide used in the substitution of the Sepharose was obtained from Sigma Chemical Company, St. Louis, Missouri, U.S.A., as were the $NADH_2$, NAD, pyruvate and heparin.

Starch for gel electrophoresis came from the Electrostarch Co., Madison, Wisconsin, U.S.A., and the Packard Instrument Company, Inc., Downers Grove, Illinois, U.S.A. supplied the 2,5-diphenyloxazole (POP) and the 1,4-bis-(2-(4-methyl-5-phenyloxazolyl))-benzene (POPOP) used in the scintillation fluid.

All other chemicals and solvents employed were of analytical reagent grade.

Protein determinations were made by the method of Lowry et al. [5], and lactate dehydrogenase activity was determined by the method of Wroblewski and La Due [6] using a Unicam SP800 recording spectrophotometer.

Sample preparation. Rats in the same stage of the oestrus cycle (metoestrus) were injected intraperitoneally after 16 h fast with either 150 μCi of [^{14}C]leucine or 450 μCi of [^{3}H]leucine, and their food was returned 4 h later. 20 h after the second injection (i.e. [^{3}H]leucine), the rats were killed by decapitation, and perfusion with cold 0.9% sodium chloride solution was performed via the inferior vena cava. The required tissues were removed and, either stored for short periods of time (2—3 days) at —10° C, or homogenized immediately in 2 volumes of cold 0.02 M sodium phosphate buffer, pH 6.8, in an overhead Ultra-Turrax homogenizer. The homogenates were centrifuged in a refrigerated L265-B ultracentrifuge at 165 000 × g for 60 min, and the clear supernatants dialysed overnight against 0.02 M sodium phosphate buffer, pH 6.8, 0.5 M sodium chloride. NADH was added to give a final concentration of approximately 100 μM immediately before chromatography.

Affinity chromatography. The oxamate substituted aminohexyl sepharose was prepared from Sepharose 4B (Pharmacia, Fine Chemicals, Uppsala, Sweden) in this laboratory, using an adaption of the methods of Cuatrecasas [7] and O'Carra and Barry [8]. Sepharose 4B was first substituted with diaminohexyl sidechains by cyanogen bromide activation, and these sidechains were subsequently reacted with oxalate to bind this competitive inhibitor of lactate dehydrogenase in the form of an oxamate analogue. Columns of the substituted Sepharose were then equilibrated with 0.02 M sodium phosphate buffer, pH 6.8, 0.5 M sodium chloride at 4°C, the temperature at which all chromatography was performed.

Just prior to sample application, buffer containing 100 μM NADH was substituted for the eluting buffer. After application of the sample, washing was continued with the NADH buffer until no further protein, as indicated by the A_{280nm} values, could be detected in the eluant. At this stage the initial buffer (i.e. without NADH) was again used to wash the column. All fractions containing activity were pooled and concentrated, initially in a stirred concentration cell using nitrogen pressure and a PM 30 ultrafiltration membrane (Amicon Corporation, Lexington, Mass., U.S.A.); final concentration to small volumes (generally less than 0.5 ml) was achieved in Minicon B15 clinical sample concentrators (Amicon Corporation).

Starch gel electrophoresis. Starch gels were prepared using 48 g of electrostarch in 440 ml of 0.03 M Tris/glycine, pH 9.0, buffer. 8 mg of heparin (sodium salt, 170 USP units/mg) were dissolved in the gel buffer as directed by the suppliers. Vertical starch gels, with 20 sample slots, were run for 3.25 h at 4°C at a constant 600 V, using a discontinuous electrode buffer system, of 0.12 M Tris/glycine, pH 9.0, for the cathode, and a 0.08 M solution of the same buffer for the anode.

The concentrated samples of lactate dehydrogenase from each tissue were run in triplicate in adjacent slots on the gel, after which the gel was cut into longitudinal strips of equal width. One of the strips for each tissue was then sliced and stained for lactate dehydrogenase activity. The regions of isoenzyme activity were excised from the corresponding positions of the remaining two strips of gel, and each of these starch blocks finely chopped and placed in a 20-ml screw top counting vial. Up to 0.8 ml of hydrogen peroxide (30% w/v) was added to each vial depending on the quantity of starch to be digested. The vials were securely capped and placed in an oven at 70°C for 12 h, after which they were allowed to cool before 15 ml of scintillator (xylene/Triton X-100 - POP, POPOP) [9] was added, mixed thoroughly, and allowed to equilibrate in the dark for several hours to ensure fluorescence had been eliminated, before counting.

Preparation of total enzyme and total protein samples for counting. The concentrated lactate dehydrogenase samples obtained from each tissue after affinity chromatography, were counted by dissolving either 0.1- or 0.2-ml aliquots directly in 3 ml of xylene/Triton scintillator.

0.1-ml aliquots of the clear supernatants obtained after centrifugation of the tissue homogenates were placed directly in the counting vials. Protein was precipitated by the addition of 0.2 ml of 10% trichloroacetic acid, and the vials were centrifuged for 5 min at 3000 × *g*. The supernatant was removed and the

precipitates washed once with diethyl ether, then dissolved in 0.1 ml of 0.2 M sodium hydroxide. Finally 3 ml of xylene/Triton scintillation fluid was added and the samples counted.

Scintillation counting. All radioactivity counting was done in a Beckman LS-250 Liquid Scintillation Counter, incorporating the automatic quench control (AQC) on the instrument. The channels chosen were the standard ^{14}C isoset provided by Beckman (range 300—490) and a narrow tritium (^{3}H) window (range 000—200) made using a variable isoset. These settings gave approximately 12% spillover of ^{14}C into the ^{3}H channel, and no spillage of ^{3}H into the ^{14}C channel. Counting was normally to 5% error or less where time permitted. Standard curves were constructed using the [^{3}H]leucine and the [^{14}C]leucine used in the experiments.

Results

A typical elution profile for the affinity chromatography step is shown in Fig. 1, which serves to illustrate the degree of purification of lactate dehydrogenase from a rat muscle extract. When samples taken from across this elution profile were electrophoresed on starch gels, and the gels then stained for lactate

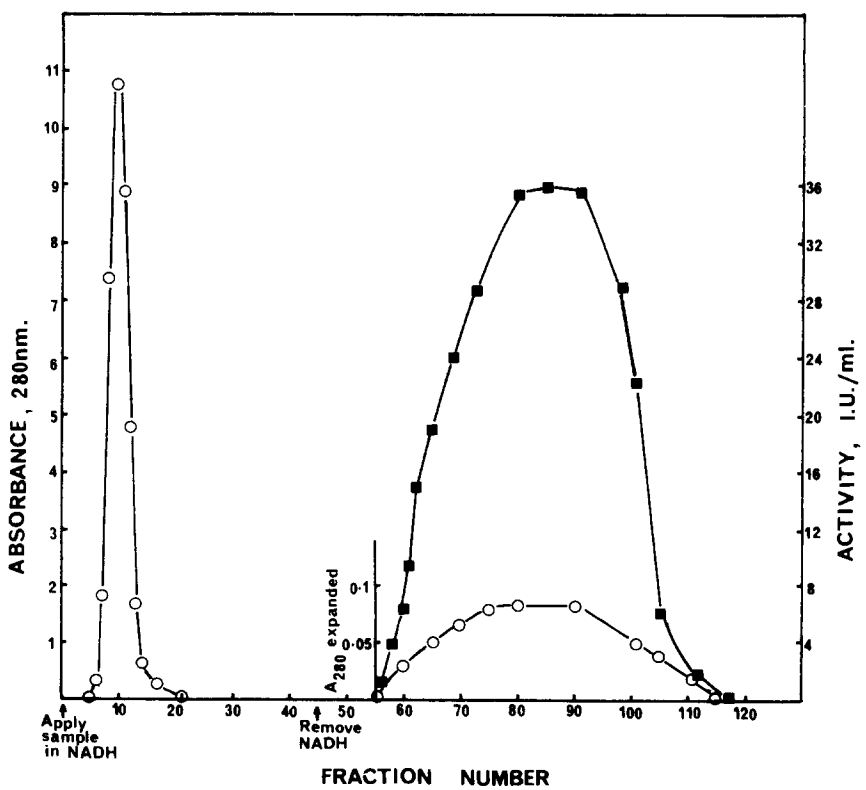

Fig. 1. Affinity chromatography of the soluble protein extract from rat muscle. Eluant was 0.02 M sodium phosphate buffer, pH 6.8, 0.5 M sodium chloride, 100 μM in NADH. The NADH was omitted from the eluant at the position indicated. ○—○—○, absorbance at 280 nm; ■—■—■, activity in I.U./ml.

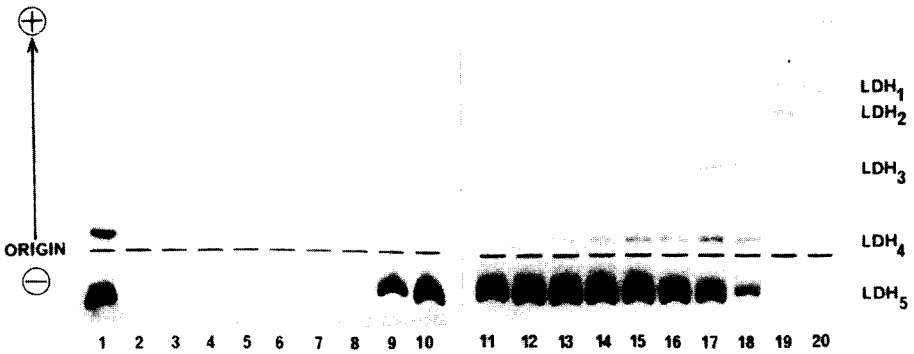

Fig. 2. Starch gel electrophoresis of lactate dehydrogenase activity in fractions collected across the elution profile during affinity chromatography of rat muscle homogenate. Numbers refer to fraction numbers as indicated in Fig. 1. Slot 1, 1/10 dilution muscle; slot 2, fraction 4; slot 3, fraction 6, slot 4, fraction 8, slot 5, fraction 10; slot 6, fraction 20; slot 7, fraction 40; slot 8, fraction 55; slot 9, fraction 60; slot 10, fraction 65; slot 11, fraction 70; slot 12, fraction 75, slot 13, fraction 80, slot 14, fraction 85; slot 15, fraction 90; slot 16, fraction 95; slot 17, fraction 100; slot 18, fraction 105; slot 19, fraction 110; slot 20, fraction 117.

dehydrogenase activity and for protein, no protein bands were observed other than in corresponding positions to the isoenzymes in fractions 50—120 (Fig. 2).

Two facts, in particular, emerge from an examination of this data. (i) The enzyme as isolated was of very high purity. The specific activity of 500 I.U./mg may be compared with the specific activities of the enzyme purified from rat liver by conventional techniques e.g. 420 I.U./mg [10] and 103 I.U./mg [11]. (ii) A difference in the isoenzyme pattern occurred in the sequential fractions from the column. Both of these aspects were characteristic of other tissue sources which were extracted by this methodology during these studies; namely liver, kidney, heart, brain, lung, pancreas, spleen, erythrocytes and reproductive tract tissues.

Having confirmed that the purification procedure met the requirements for turnover studies, in that it effected the rapid purification of this enzyme from a variety of crude tissue sources, the methodology was applied to tracing the time course of incorporation of isotope into the separate rat tissues after peritoneal injection of tritium-labelled leucine. These data are summarized for the three major tissues, liver, heart and skeletal muscle in Fig. 3. It may be seen from these data that peak values for the specific radioactivity varied both between tissues and between total protein and enzyme. Liver exhibited the highest values of specific activity in both cases (4—8 h after injection of isotope), heart displayed an early decline in enzyme radioactivity, while skeletal muscle exhibited no clear peak of specific radioactivity in the time period studied. Table I summarizes the peak specific radioactivity times for both total soluble protein and total lactate dehydrogenase in the remaining tissues studied. The variation in the peak times is again evident amongst these tissues, but with the exception of muscle, it may be seen that a time period of 20 h following injection generally encompasses the peak occurrence in all tissues.

Next, it was considered necessary to establish the extent of variability in double-isotope ratios, which was attributable to experimental design and procedure when lactate dehydrogenase and total soluble protein fractions were ex-

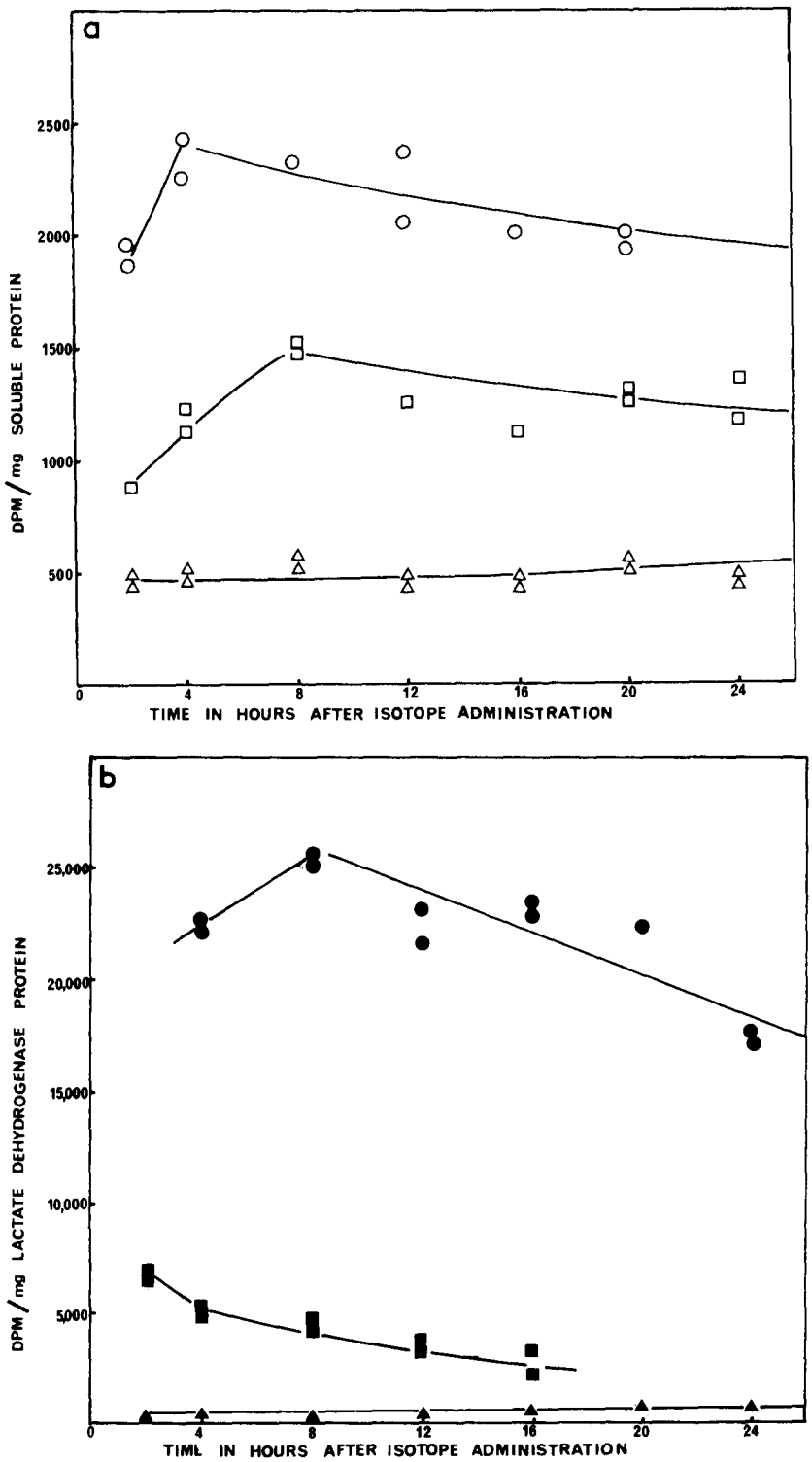

Fig. 3. Time course of labelling of total protein (a) and total lactate dehydrogenase (b) samples in rat tissues following a single peritoneal injection of 90 μCi of [^{3}H]leucine. ○, heart; □, liver; △, skeletal muscle.

TABLE I

TIMES OF OCCURRENCE OF PEAK SPECIFIC RADIOACTIVITIES IN RAT TISSUES

32 rats each received a single peritoneal injection of 90 μCi of [^{3}H]leucine after a 16 h fast. Four rats where then killed at time periods ranging from 2 to 32 h following the injection. Tissues were pooled, lactate dehydrogenase purified and duplicate samples counted, for total protein as described in the methods section, and total lactate dehydrogenase.

Tissue	Protein peak (h)	Lactate dehydrogenase peak (h)
Liver	4	8
Brain	8—12	8—12
Heart	8	2
Kidney	8—12	4
Erythrocyte	8	8
Lung	4	2
Pancreas	4	4
Spleen	16	8—12
Skeletal muscle	>32	>32
Uterus	20	20
Uterine fluid	20	20
Ovary	12—16	20
Fallopian rube	16	—
Fallopian fluid	4; >32	—

TABLE II

DOUBLE-ISOTOPE RATIOS FOR TISSUE TOTAL PROTEIN AND TOTAL LACTATE DEHYDROGENASE AFTER SIMULTANEOUS INJECTION

Two rats in metoestrus each received a simultaneous injection of 450 μCi of [^{3}H]leucine and 150 μCi of [^{14}C]leucine. The animals were killed 20 h later. For total protein estimations, the tissue homogenate from each animal was treated separately and duplicate counts of these as well as samples of the mixed homogenate supernatants were made. Lactate dehydrogenase was purified from the pooled supernatants and was counted in duplicate.

	^{3}H : ^{14}C Ratio
Injection material	2.57 ± 0.03
Total protein	
Liver	2.3 ± 0.09
Heart	2.48 ± 0.13
Kidney	2.33 ± 0.1
Brain	2.48 ± 0.17
Lung	2.4 ± 0.1
Skeletal muscle	2.4 ± 0.09
Erythrocyte	2.56 ± 0.1
Spleen	2.65 ± 0.16
Pancreas	2.29 ± 0.03
Uterus	2.28 ± 0.16
Uterine fluid	2.4 ± 0.05
Ovary	2.36 ± 0.07
Fallopian tube	2.34 ± 0.15
Fallopian fluid	2.54 ± 0.17
Total lactate dehydrogenase	
Liver	2.34 ± 0.04
Heart	2.45 ± 0.05
Kidney	2.33 ± 0.02
Brain	2.46 ± 0.01
Lung	2.32 ± 0.09
Skeletal muscle	2.26 ± 0.007
Erythrocyte	2.35 ± 0.03
Uterus	2.45 ± 0.06

TABLE III

DOUBLE-ISOTOPE RATIOS FOR TISSUE TRICHLOROACETIC ACID SUPERNATANT AFTER SIMULTANEOUS INJECTION

Two rats each received a simultaneous injection of 450 μCi of [^{3}H]leucine and 150 μCi of [^{14}C]leucine. They were killed 20 h later. Duplicate samples of the trichloroacetic acid supernatants obtained on preparation of the total protein fractions for the tissue homogenates from each rat as well as the pooled homogenates were counted.

Sample	Ratio ^{3}H : ^{14}C
Liver	2.4 ± 0.1
Heart	2.6 ± 0.1
Kidney	2.42 ± 0.15
Brain	2.46 ± 0.1
Lung	2.35 ± 0.08
Skeletal muscle	2.6 ± 0.3
Erythrocyte	2.66 ± 0.4
Spleen	2.3 ± 0.1
Pancreas	2.29 ± 0.09
Uterus	2.32 ± 0.1
Uterine fluid	92 ± 20
Ovary	12.7 ± 1.3
Fallopian tube	4.8 ± 0.4
Fallopian fluid	52.5 ± 18.0

tracted from the different tissue sources by the methodology already described. Consequently 900 μCi of [^{3}H]leucine and 300 μCi of [^{14}C]leucine were mixed, and half of the combined solution injected into each of two rats. 20 h later the animals were killed and lactate dehydrogenase isolated from those tissues where enough activity could be obtained to warrant purification. Table II shows the ^{3}H:^{14}C ratios obtained in this experiment. The trichloroacetic acid supernatant fractions of these tissues were also counted and these results are listed in Table III.

The mixture of isotopically labelled amino acid before injection counted with a ^{3}H:^{14}C ratio of 2.6. It is of significance in relation to the general applicability of the technique, that the simultaneous injection of [^{3}H]leucine and [^{14}C]leucine led to total protein and lactate dehydrogenase values for all the tissues investigated with ratios in the region 2.3—2.7. Similarly the ratios obtained for the trichloroacetic acid supernatants of all tissues (except some of the reproductive tract) gave ratios comparable with the injection material. The uterine and fallopian fluids, however, were notable as giving ratios considerably higher than that of the injected material.

Having established the general validity of the methodology in relation to enzyme purity and isotope recovery, and having surveyed the onset of decay kinetics, double-isotope experiments were set up using a 3-day and a 10-day interval between the injections of the single isotopes. The reason for employing both of these time periods lay in the fact that this combination of periods allows a broad range of half lives to be estimated, and also affords a degree of verification in estimates between the two sets of results [4,12].

Table IV lists the double-isotope ratios obtained for the total purified lactate dehydrogenase from liver for both the three day and the ten day experi-

TABLE IV

DOUBLE-ISOTOPE RATIOS FOR RAT LIVER

Five rats in metoestrus each received an intraperitoneal injection of 150 μCi of [^{14}C]leucine. This was followed either 3 or 10 days later by a similar injection of 450 μCi of [^{3}H]leucine and the animals were killed 20 h later. Liver tissue from all animals was pooled and lactate dehydrogenase purified as in the methods section. All scintillation counting was in duplicate. ^{3}H : ^{14}C ratios were corrected to assume an initial ratio of isotopes of 3.0 to allow calculations for half lives to be made by the method of Glass and Doyle [4].

Time interval	dpm ^{3}H	dpm ^{14}C	Ratio ^{3}H/^{14}C	Corrected ratio ^{3}H/^{14}C
Total protein				
3 day	108000 ± 2000	24500 ± 80	4.4 ± 0.09	5.08 ± 0.09
10 day	100900 ± 4500	10200 ± 500	9.87 ± 0.95	11.39 ± 0.05
Total lactate dehydrogenase				
3 day	61000 ± 500	16900 ± 600	3.63 ± 0.1	4.2 ± 0.1
10 day	152000 ± 1000	17900 ± 400	8.5 ± 0.13	9.81 ± 0.13

ment. Using the method of starch gel electrophoresis described earlier, an estimation of the double-isotope ratios for the individual isoenzymes was also obtained, and these values are listed in Table V for both experiments. Additionally, half lives have been estimated from the double-isotope ratios, by the method of Glass and Doyle [4], and are listed in Table VI. Both sets of time periods show a gradation of half lives for the isoenzymes, decreasing in the sequence LDH-5 > LDH-4 > LDH-3.

Discussion

The present investigation has pointed to the procedure of affinity chromatography as being highly suited to turnover studies. The technique which has been described in this paper, for example, allows the separation of the enzyme from tissue sources, rapidly, in good yield and with high purity. Indeed, in view of the importance of the latter parameter in turnover studies [12], it is worthy of comment that this one-step procedure affords an enzyme of superior specific activity to that produced in classical, multi-step, purification techniques [10,11,13].

TABLE V

DOUBLE-ISOTOPE RATIOS FOR LACTATE DEHYDROGENASE ISOENZYMES FROM RAT LIVER

Duplicate samples of the purified lactate dehydrogenase from 3- and 10-day experiments were electrophoresed on starch gels. The gels were treated as in the methods section to facilitate scintillation counting of the separate isoenzymes. The ratios have been corrected to allow calculation of half lives by the method of Glass and Doyle [4].

Time interval	Total LDH	LDH 5	LDH 4	LDH 3
3 days	4.2 ± 0.1	3.75 ± 0.2	5.92 ± 0.07	10.7 ± 1.0
10 days	9.81 ± 0.13	10.4 ± 0.06	11.7 ± 2.0	29.3 ± 6.0

TABLE VI

HALF LIVES FOR TOTAL PROTEIN AND THE ISOENZYMES OF LACTATE DEHYDROGENASE FROM RAT LIVER

Double isotope ratios calculated from the previously described 3- and 10-day interval experiments have been used to determine half life values by the method of Glass and Doyle [4].

Fraction	$t_{1/2}$ (days) 3-day interval	$t_{1/2}$ (days) 10-day interval
Total protein	2.98 ± 0.03	3.24 ± 0.05
Total lactate dehydrogenase	3.56 ± 0.09	3.75 ± 0.05
LDH 5	4.05 ± 0.2	3.53 ± 0.05
LDH 4	2.65 ± 0.02	3.3 ± 0.5
LDH 3	1.85 ± 0.09	1.8 ± 0.16

In many turnover studies, an alternative approach to the ready collection of specific protein samples has been utilized; namely, immunological precipitation [10,14,15]. Consequently, a comparative comment on these different approaches seems warranted in the present context. Granted that both methods offer the advantage of relative speed and specificity, it may be further noted that affinity chromatography offers in principle a much wider application, in that its use is not restricted by species specificity in the same manner as immunoprecipitation [14]. Additionally, since affinity chromatography utilizes the active site of the enzyme as the determinant of purification [8], rather than the surface characteristics of the molecules with antigen-antibody interactions, isolation of the enzyme by affinity chromatography should be most relevant in relation to the parameter of biological function and free from disadvantageous features of biological techniques such as coprecipitation. It is suggested however, that these two approaches to the separation of specific proteins during turnover studies (i.e. affinity chromatography and immunoprecipitation) should not be regarded as mutually exclusive alternatives; rather, because of the different parameters they represent, the comparison of results from these two procedures may well be especially valuable in furthering our understanding of the degradative factors involved in protein turnover.

In regard to the use of the double label technique in the study of protein turnover, it may be noted that this subject has been discussed at length previously [3,4,12], and that one of the assumptions of this technique is that the labelled proteins are in a process of isotopic decay at the time of death of the experimental animal. Whereas in the case of liver, this onset of decay is established as rapid [16], the extension of this technique to other tissues in the present investigation has required additional data on the time course of isotope-enzyme interactions. It seems clear from this data, that quite considerable tissue individuality occurs in regard to the incorporation phenomena, but it is also evident that a period of twenty hours encompasses the onset of decay in all major tissues except skeletal muscle. It is also clear from the results of the simultaneous injection of double label, that the present methodology is well suited to the recovery of label in all the tissues studied, and that under the experimental conditions there is little loss of tritium label outside the protein synthesis-degradation pool.

In relation to the special requirements of collection of the lactate dehydrogenase isoenzymes for isotopic assay, it should be remarked that gel electrophoresis still represents the most convenient and widely utilized resolutionary methodology for the separation of enzyme heteromorphs. Consequently, the procedure which has been described for the LDH isoenzymes in this paper is noteworthy not only in the present context, but also in relation to the more general application of similar procedures to turnover studies with other systems of multiple enzyme forms.

Finally, in relation to the actual values for half lives which have been cited in the present communication, comparison may be drawn with previous values in the literature. For the total soluble protein in rat liver, Kuehl and Sumison [10] have reported a half life of 6.6 days, Glass and Doyle [4] a value of 5.0 days and Fritz et al. [15] a figure of 2.2 days, whereas the average value in the present studies was 3.1 days. In the case of the liver lactate dehydrogenase, Kuehl and Sumison [10] calculate a value of 3.5—6.0 days, Glass and Doyle [4] a value of 6.5—7.0 days and Fritz et al. [15] a value of 19 days, while a figure of 3.6 days was arrived at in the present studies. The only previous published values for the lactate dehydrogenase isoenzymes in rat liver are those given by Fritz et al. [14,15], namely 19 and 25 days for A_4 and A_3B, respectively forms; whereas in the present study, a gradation of values was evident in the order LDH5, LDH4, LDH3: 3.7 days, 3.0 days, 1.9 days. Furthermore, from an examination of the relevant literature, it is evident that estimates of the half lives of tissue proteins vary markedly according to the particular isotopic amino acid or experimental technique chosen for the study [3,4,10,14,17]. It may be concluded from the above data, however, that the present methodology gave results which corresponded to the order of previous values in the case of total protein, but tended to give values in the lower range of previous half life determinations for the enzymes as indicated previously. It should be recognized, that much of the difference between values for liver lactate dehydrogenase in the previous [14,15] and present studies may be attributed in a large part to the different parameters of recognition in these two approaches: one would expect, for example, that the activity of the enzyme would be more sensitive, and diminish more rapidly under the influence of degradative processes in the cells than the characteristic of immunological recognition, although other factors such as the age, sex, strain, diet or environmental influences may also affect the data [15]. Again, it may be noted that the present methodology may offer the advantage of providing more sensitivity in assessing the relative degradation rates of minor isoenzymic components, since only one time point is required in comparison with the series of points which are necessary in other methodologies [10,14,15,17]. The present procedure seems to be particularly well suited to the comparison of the relative turnover rates of individual isoenzymes within the separate tissues of an animal.

Overall, then, the combination of procedures of affinity chromatography, double isotope administration and gel electrophoresis of isoenzymes appears to be substantiated as an effective approach to turnover studies, offering many advantages in relation to the high degree of purity of the protein(s) under study, the degree of time involvement, the utilization of biological activity as the parameter influenced by degradation, the broadness of application, and the

sensitivity to the comparative behaviour of multiple enzyme forms. The problems of interpretation in terms of absolute half lives (e.g. adequate compensation for compartmentation and reutilization), which are characteristic of the double label technique and other turnover methodologies are not, of course, entirely obviated by the present system. Nor does it seem to be applicable in its present form to the very slow turnover of skeletal muscle [18,19]. Nevertheless the procedure should widen the scope for turnover studies to include a broad range of phylogeny and ontogeny, and should greatly facilitate comparative studies of isoenzyme turnover in regard to important biological phenomena such as hormonal stimulation, carcinogenesis and other disease conditions.

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