Aldehyde Dehydrogenase in 2-Acetamidofluorene-Induced Rat Hepatomas

ONTOGENY AND EVIDENCE THAT THE NEW ISOENZYMES ARE NOT DUE TO NORMAL GENE DE-REPRESSION

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The pre- and post-natal ontogeny of Sprague–Dawley rat liver aldehyde dehydrogenase [aldehyde–NAD(P)⁺ oxidoreductase, EC 1.2.1.5] is described. At no time in its ontogenetic development does normal liver aldehyde dehydrogenase exhibit any of the characteristics of a series of unique aldehyde dehydrogenases that can be isolated from 2-acetamidofluorene-induced rat hepatomas. Enzyme activity is first detectable in 15-day foetal liver and gradually increases throughout pre- and post-natal development until adult activities are attained by day 49 after birth. Electrophoretically, normal aldehyde dehydrogenase, throughout its ontogeny, exists as the same single isoenzyme found in normal adult liver. Isoelectric points for two normal liver isoenzymes demonstrable by isoelectric focusing are pH5.9 and 6.0. The immunochemical properties of aldehyde dehydrogenase during its ontogeny are identical with those of normal adult liver aldehyde dehydrogenase when tested against anti-(hepatoma aldehyde dehydrogenase) serum in Ouchterlony double-diffusion tests. The results indicate that the hepatoma-specific aldehyde dehydrogenases are not the result of the de-repression of genes normally repressed in adult rat liver or in some other adult tissue.

Sugimura *et al.* (1966), Markert (1968), Potter (1969) and Uriel (1969) have all suggested that the biochemical and genetic changes occurring during the neoplastic transformation represent the return of transformed cells to a less differentiated and/or foetal state. Each of these hypotheses finds much support in the literature (Weinhouse *et al.*, 1972; Weinhouse, 1973) and are considered by many to be a general phenomenon occurring in hepatomas and perhaps in all tumours (Weinhouse *et al.*, 1972; Weinhouse, 1973).

Feinstein (1975) and Lindahl & Feinstein (1976) have demonstrated that aldehyde dehydrogenase [aldehyde–NAD(P)⁺ oxidoreductase, EC 1.2.1.5] in 2-acetamidofluorene-induced hepatomas from Sprague–Dawley rats exists as three major and several minor isoenzymes. In contrast, normal Sprague–Dawley rat liver shows a strikingly different aldehyde dehydrogenase isoenzyme pattern after gel electrophoresis or gel isoelectric focusing and possesses only one-third to one-half the detectable aldehyde dehydrogenase activity of a tumour. The hepatoma-specific aldehyde dehydrogenases differ from normal liver aldehyde dehydrogenase in sub-cellular distribution, substrate and coenzyme specificity and a variety of other biochemical and physical properties (Feinstein, 1975). Lindahl & Feinstein (1976) have purified and partially characterized the hepatoma-specific aldehyde dehydrogenases. By

using the purified hepatoma isoenzymes as antigen, an antisera against these tumour-specific species has been produced (Lindahl & Feinstein, 1976). The use of a series of purified hepatoma aldehyde dehydrogenases as antigen resulted in the production of an antisera containing two distinct antibody populations. One of these antibody populations is hepatoma-specific, whereas the other cross-reacts with normal liver aldehyde dehydrogenase.

All 2-acetamidofluorene-induced hepatomas examined to date show the same pattern of new aldehyde dehydrogenase isoenzymes and the same biochemical and immunochemical differences from normal liver aldehyde dehydrogenase. These similarities indicate that the new isoenzymes are not the result of a somatic mutation. Rather, these isoenzymes are the result of either the de-repression of a normally repressed gene(s) and/or the post-translational modification of otherwise normal enzyme species (Lindahl & Feinstein, 1976).

Whether the aldehyde dehydrogenase phenotype expressed in 2-acetamidofluorene-induced hepatomas is consistent with the hypothesis of resurgence of foetal characteristics in transformed cells is unknown. The expression of hepatoma-specific aldehyde dehydrogenase during normal liver development would indicate that the de-repression of aldehyde dehydrogenase gene(s) normally repressed in adult liver occurs during 2-acetamidofluorene-induced

hepatocarcinogenesis. The absence of hepatoma-specific aldehyde dehydrogenase during normal liver development, coupled with other characteristics of the hepatoma isoenzymes, would indicate that the aldehyde dehydrogenase phenotype expressed during hepatocarcinogenesis does not follow the 'oncogeny is blocked ontogeny' concept of Potter (1969). In an effort to clarify further the relationship between the hepatoma-specific aldehyde dehydrogenases and their normal liver counterpart, the present paper describes the pre- and post-natal ontogeny of aldehyde dehydrogenase in normal Sprague-Dawley rat liver.

Materials and Methods

Male and female Sprague-Dawley rats were obtained from Charles River Breeding Laboratories (Wilmington, MA, U.S.A.). Virgin females (110–120 days old) were pair-mated to proven fertile males. Mating was confirmed by the presence of a copulation plug (day 1) and pregnant females were housed singly in polypropylene cages. All animals were provided with Wayne Lab-blocs and water *ad libitum*.

All animals used in this ontogeny study were killed between 09:00 and 11:00h by a sharp blow to the head. Pooled whole fetuses (days 10–14), pooled foetal livers (days 15–22) collected from a single dam, or the liver of individual postnatal pups, were washed in 0.9% NaCl, weighed and frozen at -20°C until examined. Ages of postnatal animals were determined by examining cages each day for newborns. All litters used were born between midnight and 06:00h so that all ages are known to at least within 6h of birth. Hepatoma was obtained from male Sprague-Dawley rats fed with 2-acetamidofluorene (Peraino *et al.*, 1971). Each tumour was washed free of blood and cut into 1 cm^3 pieces and quick-frozen in a solid CO_2 /acetone bath. Pooled quick-frozen hepatoma was stored at -70°C until used. No changes in either the biochemical or physical properties of hepatoma aldehyde dehydrogenase due to freezing and storage have been observed.

After thawing, livers were prepared as 33% homogenates in 0.06M-sodium phosphate buffer, pH 8.5, in Potter-Elvehjem tissue homogenizers. Hepatoma was prepared in the same buffer as a 20% (w/v) homogenate. Each homogenate was sonicated for 1 min at 2.5A output with a Branson Sonifier (Heat Systems Co., Melville, NY, U.S.A.) equipped with a micro tip. The sonicated tissues were centrifuged at $48000g$ for 30 min in a Sorvall RC-5 centrifuge, after which the supernatants were drawn off for use. All operations were performed at 4°C .

Aldehyde dehydrogenase activity was determined quantitatively at 25°C by monitoring the A_{340} of

NADH produced during the oxidation of propionaldehyde in a modification of the previously described assay (Lindahl & Feinstein, 1976). The reaction mixture contained: 1.0 ml of 0.06M-sodium phosphate buffer, pH 8.5, 1.0 ml of 0.016M-NAD⁺ or -NADP⁺, 0.25 ml of 0.1M-propionaldehyde or a saturated benzaldehyde solution, 10–100 μl of enzyme and water to 3.0 ml. Enzyme was added last and the increase in A_{340} was recorded at 1 min intervals for 5 min. All appropriate corrections for substrate- and enzyme-independent changes in A_{340} were made. Activities were expressed as munits/mg of protein (1 munit = 1 nmol of NADH produced/min). Protein concentrations were determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Polyacrylamide-gel electrophoresis was performed at 4°C with the Canalco apparatus and reagents (Ames Co., Elkhart, IN, U.S.A.). Gels were stained for aldehyde dehydrogenases as described previously (Lindahl & Feinstein, 1976). Control gels were stained without added substrate to test for enzyme activity due to endogenous substrates or without coenzyme to test for aldehyde oxidase (Feinstein & Lindahl, 1973).

Analytical isoelectric focusing in polyacrylamide-gel slabs was done with the LKB (Bromma, Sweden) apparatus and methods, and the gel slabs were stained for aldehyde dehydrogenase as described previously (Feinstein, 1976). Ouchterlony double-diffusion tests, with rabbit anti-(rat hepatoma aldehyde dehydrogenase) serum as antibody source, were performed as previously described (Lindahl & Feinstein, 1976).

Results

Aldehyde dehydrogenase activity is first detectable in 15-day foetal liver (Table 1). The total detectable activity increases gradually from day 15 to birth (day 22). At birth, the total aldehyde dehydrogenase activity is approx. 20% of the normal adult value. After birth, the total aldehyde dehydrogenase activity continues to increase gradually until day 49, when the total detectable activity has reached the adult value. At weaning (21–23 days *post partum*) the total aldehyde dehydrogenase activity is 57% of the normal adult value. No significant change in the rate of enzyme activity increase is associated with weaning.

The sex of the pup had no effect on the postnatal ontogeny of aldehyde dehydrogenase. The activity of aldehyde dehydrogenase found in 2-acetamidofluorene-induced hepatomas is slightly greater than twice that found in normal Sprague-Dawley rat liver (Table 1).

Aldehyde dehydrogenase is first detectable by polyacrylamide-gel electrophoresis as a single enzyme species in 17-day foetal liver (Plate 1). As with the

EXPLANATION OF PLATE I

Polyacrylamide-gel electrophoresis of foetal rat liver aldehyde dehydrogenase

Gels were electrophoresed at 1.25 mA/gel for 90 min and stained for aldehyde dehydrogenase with propionaldehyde as substrate. The arrow indicates true aldehyde dehydrogenase in normal adult liver (N) and foetal liver (16f–22f day). All other bands in these gels appear in the absence of added aldehyde substrate and represent aldehyde-independent activity (Feinstein, 1975). The hepatoma aldehyde dehydrogenases (H) appear as a broad intensely stained band.

EXPLANATION OF PLATE 2

Polyacrylamide-gel electrophoresis of newborn rat liver aldehyde dehydrogenase

Gels were electrophoresed at 2.5 mA/gel for 90 min and stained as in Plate 1. The arrow indicates true aldehyde dehydrogenase in normal adult liver (N) and neonatal livers (1–49 day). All other bands are again considered to represent aldehyde-independent activity. Increased current not only increases the mobility of all bands, but accentuates the differences in mobility between the hepatoma (H) aldehyde dehydrogenases and the normal liver isoenzymes.

EXPLANATION OF PLATE 3

Ouchterlony double diffusions of various (a) foetal and early postnatal and (b) late postnatal rat liver aldehyde dehydrogenase

Samples were, clockwise from top: 18f-, 20f-, 22f-day foetal liver; 1-, 3- and 7-day-old neonatal liver; N, normal adult liver and H, hepatoma. Centre well contained anti-(hepatoma aldehyde dehydrogenase) serum. All wells were charged with 20 μ l samples and the gel was stained for protein with 0.1% Ponceau S. In (b) samples were, clockwise from top: 3-, 7-, 14-, 21-, 35- and 49-day-old newborn liver; N, normal adult liver and H, hepatoma. Centre well contained anti-(hepatoma aldehyde dehydrogenase) serum. Sample volumes and staining were as in (a).

Table 1. *Ontogenetic changes in total aldehyde dehydrogenase activity*
Activity is expressed as munits/mg of protein, as mean $\pm$ S.D. for the number of litters (prenatal) or individual livers (postnatal) examined, as given in parentheses.

Prenatal		Postnatal			
Day	Activity	Day	Activity	Day	Activity
10-14	Not detectable (8)	1	28.53 $\pm$ 8.79 (3)	21	66.30 $\pm$ 6.42 (3)
15	3.08 (2)	2	34.66 $\pm$ 9.17 (3)	28	73.10 $\pm$ 13.50 (3)
16	4.13 $\pm$ 2.25 (3)	3	34.92 $\pm$ 9.56 (5)	35	94.30 $\pm$ 29.82 (3)
17	7.55 $\pm$ 1.77 (3)	4	36.80 $\pm$ 11.81 (4)	42	101.10 $\pm$ 26.59 (3)
18	9.95 $\pm$ 0.81 (3)	5	36.32 $\pm$ 9.24 (4)	49	118.90 $\pm$ 25.10 (2)
19	12.11 $\pm$ 3.89 (3)	6	39.80 $\pm$ 5.18 (3)		
20	13.20 $\pm$ 2.19 (3)	7	40.92 $\pm$ 10.92 (4)	Adult	122.50 $\pm$ 10.13 (7)
21	21.13 $\pm$ 8.84 (3)	10	43.70 $\pm$ 5.71 (3)		
22	24.60 $\pm$ 5.31 (3)	14	47.77 $\pm$ 4.74 (3)	Hepatoma	261.80 $\pm$ 10.33 (3)

Fig. 1. *Polyacrylamide-gel isoelectric focusing of foetal and neonatal rat liver aldehyde dehydrogenases*
Samples (50 μ l) were focused for 4 h at 4°C and stained for aldehyde dehydrogenase with propionaldehyde as substrate. A narrow end slice of gel was used to determine pH at 5 mm intervals. In normal adult (N), foetal (day 18f, 20f, 22f-day) and neonatal (1-49-day-old) liver, the single aldehyde dehydrogenase demonstrable by gel electrophoresis is resolved into two isoenzymes with pI values of 5.9 and 6.0. The hepatoma aldehyde dehydrogenases (H) are again distinctly different, with pI values of 6.8-7.2.

spectrophotometric quantitative assays of activity, a gradual increase in activity, as judged by increasing staining intensity, occurs throughout prenatal and early postnatal life. Gel electrophoresis also indicates that adult aldehyde dehydrogenase activities are reached by 42 days *post partum* (Plate 2). Throughout its ontogeny, the enzyme activity remains electrophoretically as the same single species as is first detectable in 17-day foetal liver. This single species is also identical in mobility with the single enzyme activity found in normal adult liver. Gel electrophoresis indicates that hepatoma aldehyde dehydrogenase differs both quantitatively and qualitatively from normal liver aldehyde dehydrogenase (Plates 1 and 2). The hepatoma aldehyde dehydrogenases

exist as a series of four to five enzymic species migrating more anodally and staining much more intensely that does the single normal liver isoenzyme. Although not shown, throughout its ontogeny normal liver aldehyde dehydrogenase and the hepatoma isoenzymes reveal the same differences in substrate and coenzyme specificity after electrophoresis as described earlier (Feinstein, 1975). Normal liver aldehyde dehydrogenase, throughout its pre- and post-natal ontogeny, oxidizes propionaldehyde very readily with NAD⁺ as coenzyme and to a limited extent can oxidize propionaldehyde in the presence of NADP⁺. Normal liver aldehyde dehydrogenase oxidizes the larger, aromatic benzaldehyde only weakly, however, and only in the

presence of NAD^+ . The hepatoma aldehyde dehydrogenases can oxidize both propionaldehyde and benzaldehyde very effectively in the presence of either NAD^+ or NADP^+ .

Studies, by isoelectric focusing, of the ontogeny of normal liver aldehyde dehydrogenase indicate the same quantitative and qualitative changes as demonstrated by gel electrophoresis (Fig. 1). Qualitatively, normal liver aldehyde dehydrogenase is resolvable into two isoenzymes with pI 5.9 and 6.0. The hepatoma aldehyde dehydrogenases are again both quantitatively and qualitatively different from the normal isoenzymes. The hepatoma aldehyde dehydrogenases are resolvable into a series of intensely staining enzymic species at pI 6.8 to 7.2 (Fig. 1).

As described previously (Lindahl & Feinstein, 1976), antisera prepared in rabbits against purified hepatoma-specific aldehyde dehydrogenase cross-reacts with adult normal liver aldehyde dehydrogenase. When the anti-(hepatoma aldehyde dehydrogenase) serum is used as antibody source in immunodiffusion experiments using aldehyde dehydrogenase from livers of various foetal and early postnatal ages as antigen, a single precipitin band, identical with that obtained with adult normal liver, is observed (Plate 3). In foetal liver the precipitin band is first detectable at day 16, although a strong band is first formed with day-18 foetal liver. The precipitin band is not formed in pre-16-day foetal liver or whole foetuses. This band remains the only precipitin band detectable throughout later foetal and postnatal development, as well as in adult liver. When hepatoma is used as the source of antigen, two precipitin bands are formed. One band is continuous with the single band formed in normal adult, foetal or early postnatal liver. The other band is found only in hepatoma preparations and shows no identity with any antigen in any normal liver preparation (Plate 3).

Discussion

Both quantitative and qualitative differences in the enzymic complement of the liver as a result of the neoplastic transformation are well known (Weinhouse *et al.*, 1972; Schapira, 1973; Weinhouse, 1973). The quantitative changes may be represented as either an increase or a decrease in total enzyme activity. The majority of the qualitative modifications appear as an altered expression of enzymes that exist as multiple molecular forms; i.e. as isoenzymes. In many cases, these qualitative modifications of isoenzyme pattern are the result of expression of isoenzymes of a particular enzyme not found in normal adult liver, but commonly expressed foetal and/or neonatal liver (Weinhouse *et al.*, 1972; Schapira, 1973; Weinhouse, 1973). Several isoenzyme systems also exist in which new isoenzyme patterns expressed in hepatomas are not characteristic of normal adult

liver, but are characteristic of the normal adult pattern of some other tissues (Weinhouse *et al.*, 1972; Schapira, 1973; Weinhouse, 1973). Moreover, the deviation of the isoenzyme patterns from normal appear to be well correlated with the growth rate and degree of deviation from normal liver morphology. Rapidly growing poorly differentiated tumours show the greatest deviation from normal in their isoenzyme patterns. Slowly growing highly differentiated tumours appear more normal with respect to isoenzyme patterns (Weinhouse *et al.*, 1972; Weinhouse, 1973).

The vast majority of these enzyme-alteration studies have utilized the series of hepatomas developed by Morris (1963, 1965) and carried by serial transplantation in Buffalo rats. None of these tumours was originally induced by 2-acetamidofluorene, and studies of the biochemical changes that occur in hepatomas induced by this aromatic amine are generally lacking. Changes in the total non-histone chromosomal protein fraction of hepatocellular carcinomas induced by 2-acetamidofluorene have been described by Becker (1976). Almost every tumour studied by Becker (1976) has appeared to possess its own unique pattern of qualitative non-histone chromosomal protein changes.

Berg & Christofferson (1974) have reported an increase in the specific activity of β -glucuronidase and acid deoxyribonuclease in rat liver parenchymal cells after 2 months exposure to 2-acetamidofluorene. These observations indicate that 2-acetamidofluorene may induce quantitative changes in the activity of genes normally expressed in adult liver. Silber *et al.* (1975) have reported that the aldolase phenotype of 2-acetamidofluorene-induced rat hepatomas is identical with that for aldolase in Morris hepatomas. As with the Morris tumours, the 2-acetamidofluorene-induced hepatomas show a resurgence of foetal aldolase activity. Silber *et al.* (1975) also observed that the new aldolase phenotype is expressed before any histological modifications typical of a hepatoma are detectable. Harada *et al.* (1976) have described a similar series of changes in γ -glutamyl transpeptidase (EC 6.3.2.3) activity during *N*-hydroxyacetamidofluorene-induced hepatocarcinogenesis. Tumour γ -glutamyl transpeptidase activity is 7–12 times the activity of normal liver γ -glutamyl transpeptidase and most of the increased activity is electrophoretically identical with foetal liver γ -glutamyl transpeptidase. The work of Silber *et al.* (1975) and Harada *et al.* (1976) indicate that 2-acetamidofluorene-induced hepatocarcinogenesis can lead to the de-repression of genes normally repressed in adult liver.

The ontogenetic changes in normal liver aldehyde dehydrogenase activity as studied here by changes in total enzyme activity, gel electrophoresis and gel isoelectric focusing and immunochemistry, confirm

and expand the observations of Pikkarainen (1971), that the total aldehyde dehydrogenase activity gradually increases during development. More importantly, however, the results reported here indicate that at no time during its ontogenetic development does Sprague-Dawley normal rat liver aldehyde dehydrogenase, by any criteria used, appear identical with or even similar to hepatoma-specific aldehyde dehydrogenase.

In addition to the fact that the hepatoma-specific aldehyde dehydrogenases are not foetal liver aldehyde dehydrogenase, the hepatoma isoenzymes are not the result of de-repression of genes normally expressed in some other adult tissue (Lindahl & Feinstein, 1976). Brain is the only other adult rat tissue that possesses significant aldehyde dehydrogenase activity and it has previously been demonstrated that the hepatoma-specific aldehyde dehydrogenases are not identical with or similar to brain aldehyde dehydrogenase (Lindahl & Feinstein, 1976). Moreover, no hepatoma-specific aldehyde dehydrogenases are detectable during liver regeneration after partial hepatectomy (Feinstein, 1975). Also, many of the phenotypic changes that occur during neoplasia also occur in other pathological conditions (Schapira *et al.*, 1975); the hepatoma aldehyde dehydrogenases are expressed only after exposure of the liver to carcinogen (Feinstein, 1975; Feinstein *et al.*, 1976). Finally, in contrast with most other phenotypic modifications during the neoplastic transformation, neither the total aldehyde dehydrogenase activity nor isoenzyme-pattern changes are correlated with the degree of deviation from normal liver morphology that any tumour expresses (Feinstein, 1975; Feinstein *et al.*, 1976).

However, as with most phenotypic isoenzyme changes that resemble the foetal condition, the hepatoma-specific aldehyde dehydrogenases are detectable before a tumour is histologically identifiable (Feinstein *et al.*, 1976). In addition, at least three chemical carcinogens, 2-acetamidofluorene, 4-dimethylaminoazobenzene and ethionine, appear capable of causing expression of the hepatoma-specific aldehyde dehydrogenase phenotype (Feinstein *et al.*, 1976).

The totality of observations of the aldehyde dehydrogenase phenotype expressed in 2-acetamidofluorene- or 4-dimethylaminoazobenzene-induced hepatomas indicate that if carcinogen-induced gene de-repression is responsible, wholly or in part, for the tumour-specific aldehyde dehydrogenase phenotype, the newly de-repressed gene(s) are not those normally

expressed during pre- or post-natal liver development or in some other normal adult tissue. Feinstein *et al.* (1976) have proposed that the hepatoma-specific aldehyde dehydrogenase phenotype is the result of de-repression of an 'archeogene'. Whether this suggestion is a viable one will first require the demonstration that the new aldehyde dehydrogenases are not the result of post-translational modification of normal liver aldehyde dehydrogenase owing to carcinogen-induced alterations in another component(s) of the hepatic metabolic machinery.

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References

- Becker, F. F. (1976) *Cancer Res.* **36**, 2563-2566
Berg, T. & Christofferson, T. (1974) *Biochem. Pharmacol.* **23**, 3323-3329
Feinstein, R. N. (1975) in *The Isozymes* (Markert, C. L., ed.), vol. 3, pp. 969-986, Academic Press, New York
Feinstein, R. N. (1976) *Anal. Biochem.* **72**, 533-538
Feinstein, R. N. & Lindahl, R. (1973) *Anal. Biochem.* **56**, 353-360
Feinstein, R. N., Fry, R. J. M., Cameron, E. C., Peraino, C. & Morris, H. P. (1976) *Proc. Soc. Exp. Biol. Med.* **152**, 463-468
Harada, M., Okabe, K., Shibata, K., Masuda, H., Miyata, K. & Enomoto, M. (1976) *Acta Histochem. Cytochem.* **9**, 168-179
Lindahl, R. & Feinstein, R. N. (1976) *Biochim. Biophys. Acta* **452**, 345-355
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275
Markert, C. L. (1968) *Cancer Res.* **28**, 1908-1914
Morris, H. P. (1963) *Prog. Exp. Tumor Res.* **3**, 370-411
Morris, H. P. (1965) *Adv. Cancer Res.* **9**, 227-302
Peraino, C., Fry, R. J. M. & Staffeldt, E. (1971) *Cancer Res.* **31**, 1506-1512
Pikkarainen, P. H. (1971) *Ann. Med. Exp. Biol. Fenn.* **49**, 151-156
Potter, V. R. (1969) *Can. Cancer Conf.* **8**, 9-30
Schapira, F. (1973) *Adv. Cancer Res.* **18**, 77-153
Schapira, F., Hatzfield, A. & Weber, A. (1975) in *The Isozymes* (Markert, C. L., ed.), vol. 3, pp. 987-1003, Academic Press, New York
Silber, D. A., Chicinska, E., Rabczynski, J. & Kochman, M. (1975) *Int. J. Cancer* **16**, 675-681
Sugimura, T., Matsushima, T., Kawachi, T., Hirata, Y. & Kawabe, S. (1966) *GANN Monogr.* **1**, 143-149
Uriel, J. (1969) *Pathol. Biol.* **17**, 877-884
Weinhouse, S., Shatton, J. B., Criss, W. E. & Morris, H. P. (1972) *Biochemie* **54**, 685-693
Weinhouse, S. (1973) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **32**, 2162-2167