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Electrophoretic Purification and Characterization of Human NADH-Glutamate Dehydrogenase Redox Cycle Isoenzymes Synthesizing Nongenetic Code-Based RNA Enzyme

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Abstract

NADH-glutamate dehydrogenase (GDH) is active in human tissues, and is chromatographically purified, and studied because it participates in synthesizing glutamate, a neurotransmitter. But chromatography dissociates the GDH isoenzymes that synthesize nongenetic code-based RNA enzymes degrading superfluous mRNAs thereby aligning the cellular reactions with the environment of the organism. The aim was to electrophoretically purify human hexameric GDH isoenzymes and to characterize their RNA enzyme synthetic activity as in plants. The outcome could be innovative in chemical dependency diagnosis and management. Multi metrix electrophoresis including free solution isoelectric focusing, and through polyacrylamide and agarose gels were deployed to purify the redox cycle isoenzymes of laryngeal GDH, and to assay their RNA enzyme synthetic activities. The laryngeal GDH displayed the 28 binomial isoenzymes typical of higher organisms. Isoelectric focusing purification produced pure GDH. Redox cycle assays of the GDH isoenzymes produced RNA enzymes that degraded human stomach total RNA. In the reaction mechanism, the Schiff-base intermediate complex between α-ketoglutarate and GDH is the target of nucleophiles, resulting to the disruption of synthesis of glutamate, and RNA enzyme. The strongest nucleophiles are the psychoactive alkaloids of tobacco, cocaine, opium poppy, cannabis smoke because they are capable of reacting with GDH Schiff base intermediate to stimulate synthesis of aberrant RNA enzymes that degrade cohorts of mRNAs thereby changing the biochemical pathways and exacerbating drug overdose and chemical dependency. Electrophoretic purification,

and characterization of the RNA enzyme synthetic activity set the forecourt for innovative application of GDH redox cycles in the diagnostic management of chemical dependency.

Keywords

GDH Electrophoretic Enzymology, Chromatographic GDH,
Total RNA-RNA Enzyme Complex, mRNA Cohorts, Chemical Dependency

1. Introduction

NADH-glutamate dehydrogenase (GDH, EC 1.4.1.2) is important in the transformations of glutamate in human tissues including in the central nervous system [1]; in anterolateral temporal neocortical and hippocampal tissues neurotransmission metabolism [2]; in neurons and astrocytes *de novo* synthesis of glutamate [3]; in pancreatic β-cells, and hepatocytes during glucose homeostasis, and insulin secretion [4]; and testicules [5] because it catalyzes the reductive amination of α-ketoglutarate (α-KG) to glutamate, an excitatory neurotransmitter. GDH is a complex mix of hexameric isoenzymes in human tissues [6] [7]; it was chromatographically purified as its dissociated subunit polypeptides [7] [8], but the physical chemistry of the hexamer was studied [9]. GDH is encoded by two nonallelic genes (GHD_1 and GDH_2) with GDH_1 encoding the more acidic polypeptides (**a**) and (***α***), being heterozygous and codominant; and GDH_2 encoding the less acidic polypeptide (***β***) being homozygous [10]. The binomial distribution of the three types of polypeptides gives rise to the complex system of hexameric isoenzymes [11] [12]. The purpose of this research project in parts was to understand the enzymology and molecular chemistry of the human hexameric isoenzymes of GDH in order to widen their potential biotechnological applications for the improvement of human health. Whereas GDH hexameric isoenzymes have been studied extensively in higher plants [13], they have not been so studied in mammalian cells and tissues. The catalytic mechanism of the enzyme involves the formation of a GDH-linked Schiff base intermediate between α-KG and the ε-NH_2 group of the Lys residue in the enzyme's active site [14]. After protonation, the Schiff base nitrogen is the target site of the action of nucleophiles including water molecule, NH_4^+, xenobiotics, ribonucleoside triphosphates, heavy metal ions, amino acids, carbohydrates, fatty acids etc; and of the isomerization phenomenon of the enzyme because the binding of xenobiotics (alkaloids, mineral ions, pyrans, amines, etc) to the Schiff base nitrogen inactivates it by formation of GDH-linked substituted imine dead-end complexes that are degraded to catalytically aberrant fragments [11] [14] [15]. *De novo* translation produces new pools of GDH polypeptides that are considerably different from the degraded pool thereby creating the hexameric isomerization phenomenon [14]. In addition to the reversible reductive amination of α-KG to L-glu, the GDH hexameric isoenzymes engage in the cyclical synthesis (**Figure**

1) of template-independent plus-RNA enzyme in the amination direction and minus-RNA enzyme in the oxidative deamination direction [16]. The GDH-synthesized nongenetic code-based RNA enzymes integrate/discriminate the biochemical pathways by degrading sick superfluous mRNAs thereby aligning the developmental and metabolic demands of cells, tissues, whole organism with the internal and external environmental conditions of the organism; meaning that every time the environment of the cells, tissues, or the entire organism changes, GDH synthesizes new panels of nongenetic code-based RNA enzymes which then degrade the unnecessary and superfluous mRNAs, rRNAs, transfer RNAs etc of the previous environment [17]. The nongenetic code-based RNA is electrostatically more stable than genetic code-based RNAs [18], therefore when an mRNA aligns with its homologous nongenetic code-based RNA enzyme, the mRNA suffers degradation [17]. Also, nongenetic code RNA enzyme degrades structurally related cohorts of mRNAs [16] [17] [18]. Wherefore, an aim of this study was to apply the GDH redox cycle [16] that we garnered from plant systems research programs (**Figure 1**) towards the expanded understanding of GDH transactions in human cells and tissues especially in diabetic, alcoholism, and narcotic chemical dependencies; because psychoactive chemicals including cannabinoids; opioids, cocaine, and tobacco alkaloids, N-nitrosoamines, alcohol, heterocyclic amines etc [19] [20] [21] are strong nucleophiles that are potentially capable of reacting with GDH-linked Schiff base intermediate to produce substituted imine dead-end complexes, that are destined for proteolytic degradation [14]. Diabetes, alcoholism, and chemical dependency are technologically inexplicable medical and social phenomena and pandemics because they still cause more than half a million deaths [22] [23].

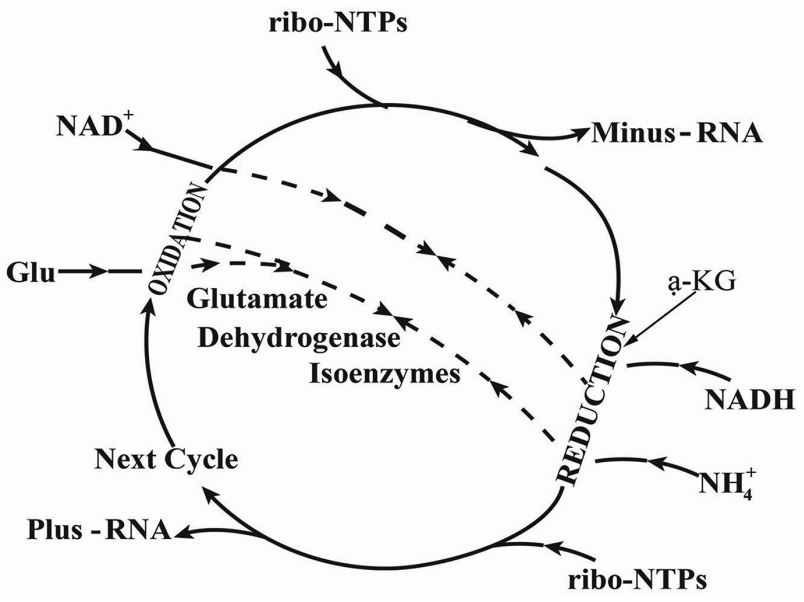

Figure 1. Redox cycles of NADH-glutamate dehydrogenase isoenzymes: For the nonreversible synthesis of nongenetic code-based plus-RNA enzyme in the amination, and minus-RNA enzyme in the deamination direction.

But dissociated GDH subunit polypeptides are unable to polymerize the ribo-NTPs to RNA. Methods for purification of human GDH have been chromatographic in chemistry [8] [9] [24] [25]. There are non-chiotropic chromatographic methods for the purification of the undessociated GDH hexameric isoenzymes from mammalian tissues. Therefore, cyclical redox synthesis of nongenetic code-based plus-RNA in the amination, but minus-RNA in the deamination direction calls for the development of gentle methods for purifying the intact hexameric isoenzymes. Crude extracts from liver, brain, and heart were purified by passing them through columns of derivatized stationary phases/matrixes (cellulose, hydrazide gel, Sepharose, hydroxyapatite etc). Each chromatographic matrix required a special buffer solution in order for the GDH to be differentially eluted, thus exposing the GDH subunit polypeptides to the risk of denaturation [6] [7] [26] [27] [28]. Accordingly, all the research programs that proclaimed the oxidative deamination of glutamate withheld the GDH redox cycle isoenzyme distribution patterns on polyacrylamide gel because the subunit polypeptides were degraded during chromatographic purification. GDH is an old enzyme in biochemistry [26], but the regulation of biochemical pathways by nongenetic code-based RNA enzyme is only beginning to be understood [17] [18].

The convergent aim of these research programs was to reset the GDH biochemical story line and forecourt so that its redox cycles of hexameric isoenzymes can be applied towards the innovative management of diabetes, alcoholism, and nicotine, opioid, cannabinoid, cocaine abuse pandemics. Hereunder, we show that electrophoresis purifies human laryngeal GDH hexameric isoenzymes; they synthesize nongenetic code-based RNA enzymes as in higher plants, and that they degrade homologous superfluous human total RNA, with potential activities to modify the biochemical pathways from the mRNA level.

2. Materials and Methods

2.1. Free Solution Isoelectric Focusing

Normal human laryngeal epithelial cell culture (120,000 cells) from LifeLine Cell Technology, Maryland, USA was homogenized in 80 mL of 15 mM Tris-HCl buffer solution, pH 7.5, containing 20 µL β-mercaptoethanol, 2 U DNase 1, and 1 U RNase A for 1 min at maximum speed. The cell culture was used as received without further culturing. The homogenate was left to stand at room temperature for 30 min for DNA and RNA to be degraded. Protein precipitated by solid $(NH_4)_2SO_4$ between 20% and 55% saturation of the homogenate was collected by centrifugation (10,000 x g, 30 min, 5˚C). Pellet was resuspended in minimum volume of 15 mM Tris-HCl pH 7.5 buffer solution, and dialyzed in 5 L of 15 mM Tris-HCl buffer solution pH 8.5; with three changes of the buffer solution over a period of 36 h.

The dialyzed extract was made up to 50 mL with 10 mM Tris-HCl buffer pH 7.5, and subjected to Rotofor (Bio-Rad, Hercules, CA) isoelectric focusing frac-

tionation [29]. Rotofor fractions were dialyzed in 5 L of 10 mM Tris-HCl buffer solution pH 8.5 also at 5˚C; with three changes of the buffer over a period of 36 h to remove the ampholyte and urea.

2.2. Polyacrylamide Gel Electrophoresis

Aliquots (100 µL) of the dialyzed Rotofor fractions were subjected to Laemmli [30] SDS 12% polyacrylamide gel electrophoresis (PAGE) (100 V, 13 h, 4˚C) to remove other proteins. Protean II xi electrophoresis cell (Bio-Rad, Hercules, CA) was used. The electrophoresed gel was washed three times with 0.15 mM Tris base at 5˚C to remove the SDS. Gel was thereafter stained with L-glutamate-NAD^+-phenazine methosulfate-tetrazolium blue reagent [10] at room temperature. The GDH redox cycle isoenzyme distribution pattern was photo-documented. Rotofor purification was repeated many times with extractions from 70,000 to 200,000 laryngeal epithelial cells; all the GDH redox cycle isoenzymes obtained displayed identical distribution patterns on polyacrylamide gel.

Aliquots (250 µL) of the dialyzed Rotofor fractions were subjected to native 7.5% PAGE (100 volts, 16 h, 4˚C) to remove contaminants (low molecular weight proteins and nucleic acids). Protean II xi electrophoresis cell (Bio-Rad, Hercules, CA) was used. GDH isoenzymes were eluted from the electrophoresed gel at subzero temperature using whole gel eluter (Bio-Rad). The whole gel fractions were applied for the synthesis of nongenetic code-based RNA. Control native 7.5% PAGE was also conducted, the electrophoresed gel was stained with tetrazolium reagent in order to confirm and locate the GDH redox cycle isoenzymes [29]. Control extractions of GDH were performed as described above but without DNase and RNase treatments.

2.3. Preparative Scale Polyacrylamide Gel Purification

For the purification of GDH polypeptides, Rotofor fractions containing GDH redox cycle isoenzymes (fractions 3 - 8) were combined, and precipitated with solid $(NH_4)_2SO_4$ to 55% saturation. Resulting pellet was suspended in 0.5 mM Tris-HCl buffer solution pH 8.5; and dialyzed exhaustively at 5˚C to remove the salt. The concentrated GDH isoenzyme solution was made 0.05% (vol./wt.) with SDS, warmed at 55˚C for 5 min in order to monomerize the hexameric isoenzymes, then loaded onto the SDS-12% PAG in Bio-Rad model 491 Prep Cell, and electrophoresed (100 volts) at 4˚C. Fraction collection was at the rate of 20 min per fraction of 3.5 mL. Protein contents of fractions were precipitated by saturating fractions to 55% with solid $(NH_4)_2SO_4$, followed by centrifugation (10,000 x g, 30 min, 5˚C). The protein pellet was dissolved in 0.1 mL of 10 mM Tris base solution and dialyzed to remove the $(NH_4)_2SO_4$. One half of the dialyzed protein was analyzed by spectrophotometry for GDH amination activity [29]; and the remaining half was used for SDS 12% PAGE (Bio-Rad Protean II xi electrophoresis cell), and electrophoresed until the blue marker dye was at the bottom of the gel. The electrophoresed gel was stained with Bio-Rads (Hercules, CA, USA) silver reagent to visualize the protein bands [31], and photo-documented.

2.4. Nongenetic Code-Based RNA Synthesis

For RNA synthetic activity of GDH, mini-whole gel eluted GDH redox cycle isoenzymes from fractions 3 to 8 were combined to give three groups as follows:

Group 1: acidic isoenzymes: combined fractions 3, and 4.

Group 2: mildly acidic isoenzymes: combined fractions 5, and 6.

Group 3: neutral isoenzymes: combined fractions 7, and 8.

Substrate cocktail per assay was prepared by adding the four ribo-NTPs (0.6 mmol each), NH_4Cl (100 µmol), α-KG (50 µmol), and NADH (0.2 µmol), DNase 1 (1 U), actinomycin D (2 µg), RNase inhibitor (10 U), the final volume made up to 1 mL with 0.1 M Tris-HCl buffer solution pH 8.0. The reaction was started by adding 1.0 mL of GDH isoenzyme solution, followed by incubation at 16˚C overnight in chilled circulating water bath. Each group of GDH isoenzymes contained about 5 µg protein per mL. Protein contents were determined using the folin-phenol reagent, and with bovine serum albumen as standard [32]. RNA synthesis was stopped by phenol-chloroform (pH 5.5) extraction of the enzyme. RNA was precipitated with ethanol, air-dried briefly, and dissolved in 40 µL of molecular biology quality water. Assays were carried out in duplicate to verify reproducibility of the results.

2.5. Degradation of Total RNA by Nongenetic Code-Based RNA Enzyme

For the degradation of total RNA, 10 µg of normal human stomach total RNA (BioChain Inc., California) was added to 10 µL of each of the three groups of RNA enzymes synthesized by laryngeal GDH redox cycle isoenzymes, and the total volume was made up to 50 µL with 10 mM Tris-HCl buffer solution pH 7.5. A control with GDH-synthesized RNA enzyme but without total RNA was set up for each GDH-synthesized RNA enzyme. The six reactions were thermo-cycled (Bio-Rad T100 thermal cycler) as described before [18]; pre-heat (96˚C, 30 sec), then 40 cycles of cool (5˚C, 1 min), and warm (37˚C, 2 min). At the end of thermocycling, the reaction was held at 5˚C. The reaction products were visualized by electrophoresing (70 volts) 15 µL of the thermo-cycled reaction solution through 1% agarose gel in 1xMOPS buffer solution with formaldehyde. The thermo-cycled reaction products were not heat-denatured before the agarose gel electrophoresis (Bio-Rad sub-cell GT cell). The electrophoresed gel was stained with ethidium bromide solution, and photo-documented.

3. Results

3.1. The Electrophoretically Purified Human GDH Redox Cycle Isoenzymes

Human NADH-GDH electrophoretic enzymology started by homogenization of the tissue with buffer solution containing DNase 1 and RNase A in order to assure the removal of all genetic code-based DNA and RNA from the crude extract. However, the NADH-GDH redox cycle isoenzyme distribution pattern

obtained when the extraction buffer solution contained DNase 1 and RNase A was same as when the extraction buffer solution did not contain DNase 1 and RNase A (**Figure 2**). Therefore, the endogenous DNases and RNases of the human cells were sufficient to degrade the genetic code-based DNA and RNA during the extraction of GDH. In the extraction of NADH-GDH from higher plants, it was necessary to add RNase A and DNase 1 to the extraction buffer solution in order to assure removal of genetic code-based RNA and DNA from the purified enzyme [13]. The binomial distribution pattern of the 28 NADH-GDH redox cycle isoenzymes of laryngeal epithelial cells (**Figure 2**) did not contain any genetic code-based RNA or DNA. Human NADH-GDH enzymology (**Figure 2**) harmonized perfectly with the GDH genetic structure of mammals and of higher plants [12] [13] [29]. GDH is made up of two nonallelic genes (GDH_1, and GDH_2) with GDH_1 encoding the more acidic polypeptides (a), and (α) being heterozygous and codominant; and GDH_2 encoding the less acidic polypeptide (β) being homozygous [10]. The binomial combination of the three types of polypeptides gives rise to the complex system of hexameric isoenzymes [13]. Chromatographic physicochemistry did not as elegantly resolve the complex isoenzyme system to biochemically active hexameric isoenzymes [2] [6] [24] [25] [28]. The pI values of the Rotofor chambers 3, 4, 5, 6, 7, and 8 in which the laryngeal GDH isoenzymes focused were 3.5, 3.8, 4.6, 5.6, 6.8, and 7.2 respectively, thus broadly dividing the isoenzymes into acidic, mildly acidic, and neutral groups that are suitable for molecular chemistry experimentations. The redox isoenzyme compositions of the acidic isoenzymes were different from those of

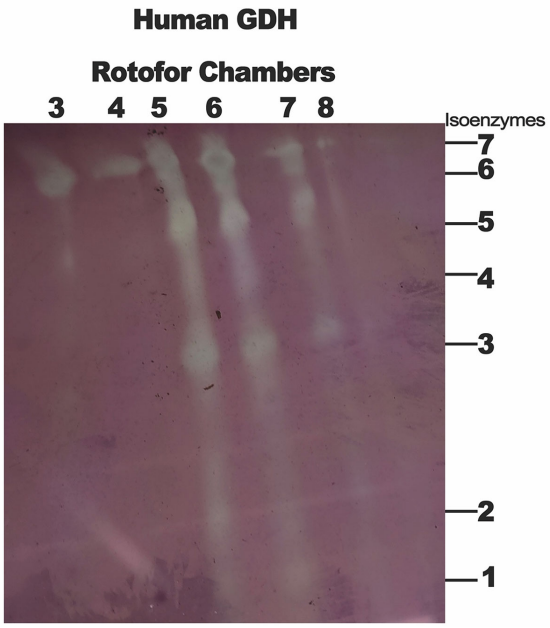

Figure 2. Glutamate dehydrogenase redox cycle isoenzymes electrophoretically purified (free solution isoelectric focusing) from human laryngeal epithelial cells. The extraction buffer solution did not contain RNase A and DNase 1. Rotofor chambers 3, 4, 5, 6, 7, and 8 in which the laryngeal GDH isoenzymes focused were pI values of 3.5, 3.8, 4.6, 5.6, 6.8, and 7.2 respectively.

the mildly acidic isoenzymes, which in turn were different from those of the neutral isoenzymes (**Figure 2**). Other chambers of the Rotofor cell contained negligible NADH-GDH hexameric isoenzyme activity. Therefore, free solution isoelectric focusing purification produced a highly purified and optimum yield of NADH-GDH redox cycle isoenzymes that are concentrated and focused into a few of the Rotofor chambers. The compactness of each hexameric isoenzyme (**Figure 2**) was testament that the NADH-GDH subunit polypeptides did not suffer degradation fragmentation during purification. The NADH-GDH hexameric isoenzymes on the 12% PAG landscape (**Figure 2**) did not suffer dissociation because they were not heat-treated prior to the 12% PAGE, which was conducted at 5˚C.

The first transit in the pharmacokinetics of cocaine, tobacco, opium, or cannabis smoke condensate is on the laryngeal epithelial lining on the journey of the smoke to the lungs, stomach, and blood stream [19]. Therefore, the laryngeal NADH-GDH is the first site of the action of psychoactive chemicals, making it imperative as an important experimental tissue for the analysis of the biochemical sequence of chemical dependency.

Purification of GDH polypeptides from Rotofor-purified redox cycle isoenzymes showed that apart from the void volume of the Prep Cell, the fractions were active in the reductive amination of α-KG; meaning that Rotofor fractions 3 to 8 contained the laryngeal NADH-GDH. The twin acidic polypeptides (**a**), and (***α***) of about 46 kDa molecular weight were eluted in fractions 60 - 75 from the Prep Cell (**Figure 3**). The less acidic polypeptide (***β***) of about 66 kDa molecular weight was eluted in fractions 105 - 126, after which no other polypeptides were present in the Prep Cell (**Figure 3**). Therefore, electrophoresis gave a purer preparation of undegraded NADH-GDH compared with chromatographic GDH [6] [8].

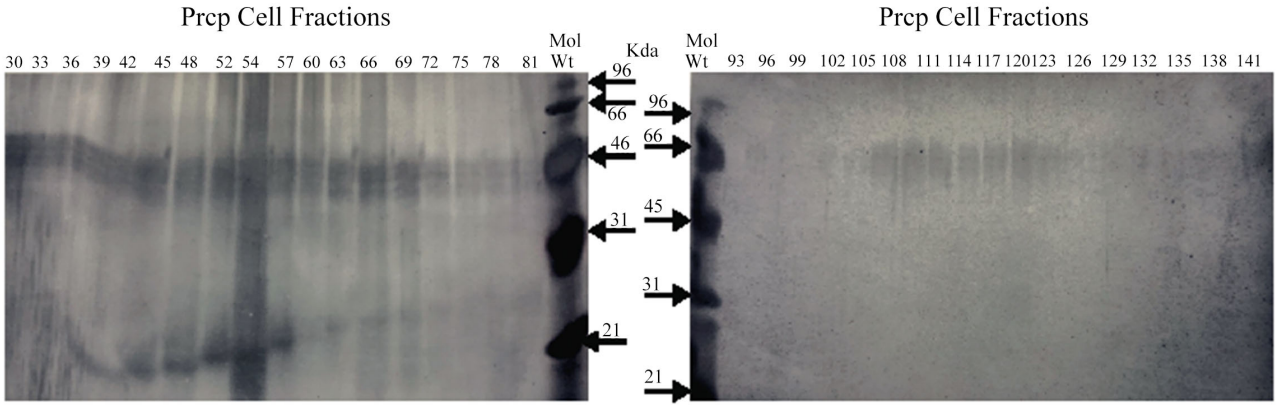

Figure 3. Protein profiles of the Prep Cell-purified human laryngeal epithelial GDH. The Rotofor fractions containing the GDH redox cycle isoenzymes were combined and purified through SDS-12% PAG of Bio-Rad's model 491 Prep Cell. Fractions from the Prep Cell were prepared and electrophoresed SDS 12% PAG of Bio-Rad's Protean II Cells. The electrophoresed gels were silver stained with Bio-Rad's silver reagent. Fractions 1 - 20 were the void volume; fractions 21 - 57 contained the degraded polypeptides of GDH; fractions 60 - 75 contained the (a), and (*α*) polypeptides; fractions 105 - 124 contained the (*β*) polypeptide of GDH. The molecular weight markers were phosphorylase b (97 KDa), BSA (69 KDa), ovalbumin (45 KDa), carbonic anhydrase (30 KDa), lysozyme (14 KDa).

NADH-GDH polypeptides are regularly degraded *in vivo* [14] after they have formed stable GDH-linked substituted imine dead-end complexes with strong nucleophilic drug molecules, xenobiotics etc. Such GDH-linked substituted imine dead-end complexes lack redox cycle activity. Much of the anomalous apparent Michaelis-Menten amination constants (K_m and V_{max}) detected in Prep Cell fractions 25 - 57 were attributable to fragmented polypeptides of GDH. The fragmentation of the GDH polypeptides detected in fractions 25 - 57 was not caused by electrophoretic purification process. In higher plants, such *in vivo* degraded GDH polypeptides were specifically detected by Western analysis using anti-GDH antibody, and they were lower molecular weight polypeptides than the polypeptides of GDH [14] [33] [34].

3.2. Degradation of Human Total RNA by GDH-Synthesized RNA Enzyme

Human stomach total RNA (substrate) was completely degraded by the nongenetic code-based RNA enzyme synthesized by laryngeal epidermal cells GDH redox cycle isoenzymes (**Figure 4**) similar to the degradation of higher plant total RNA by RNA enzyme [17] [18]. RNA enzyme synthesized by laryngeal GDH spanned a wide range of molecular weight from 25 bases to 20,000 bases long (**Figure 4**). In the chemical reaction mechanism [17], GDH synthesized nongenetic code-based RNA enzyme is guided by the flanking nonhomologous sequences to bind to its target homologous genetic code-based RNAs (rRNA, microRNA, transfer RNA, mRNA) as substrate, followed by the electromagnetic degradation of the genetic code-based RNA, the lesser stable of the two different electromagnetic kinds of RNA [18]. The enzyme-product (transformation of total RNA-nongenetic code RNA enzyme) complex was captured live on brief electrophoresis on MOPS-agarose gel before it dissociated (**Figure 4**). The band for the enzyme-product complex was momentarily migrating at a slower rate than the bands for total RNA (substrate), and the GDH-synthesized nongenetic code RNA enzyme (**Figure 4**). Because of the structural constraints imposed on it by genetic code, total RNA is not electrothermally as stable as nongenetic code-based RNA enzyme synthesized by GDH redox isoenzymes [18]. GDH-synthesized RNA enzymes are not double-stranded, the interactions between GDH-synthesized RNA and total RNA are not via base pairing, the vast majority of GDH-synthesized RNA enzymes are longer than 25 nucleotides, the synthesis of nongenetic code RNA enzyme by GDH is not initiated nor terminated by any kind of primers, therefore the chemical mechanism of degradation of total RNA by GDH-synthesized RNA enzyme is not by RNAi double-stranded ATP-dependent cleavage [35] [36] [37]. Also, the degradation of genetic code-based RNA by nongenetic code-based RNA enzyme is not by depurination or depyrimidination [17]. Genetic code-based RNA abundance is regulated post-transcription by nongenetic code-based RNA enzyme synthesized by NADH-GDH (**Figure 4**).

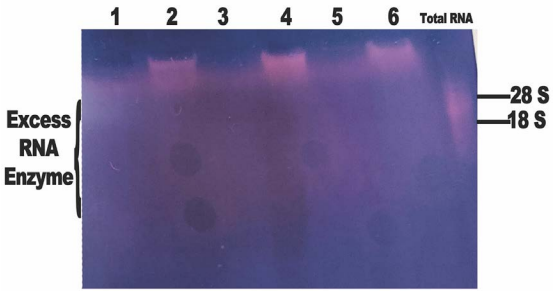

Figure 4. Total RNA-RNA enzyme complexes. Degradation of human total RNA by nongenetic code-based RNA enzymes synthesized by human GDH redox cycle isoenzymes. Laryngeal GDH redox cycle isoenzymes in micro tubes (**1**) acidic (pI 3.5 - 3.8); (**3**) mildly acidic (pI 4.6 - 5.6); (**5**) neutral (pI 6.8 - 7.2) electrophoretically purified were made to synthesize RNA enzyme. Stomach total RNA as substrate was added to micro tubes (**2**) containing the RNA enzyme synthesized by the acidic GDH isoenzymes; (**4**) containing the RNA enzyme synthesized by the mildly acidic GDH isoenzymes; (**6**) containing the RNA enzyme synthesized by the neutral GDH isoenzymes; in final volume made up to 50 μL with 0.05 M Tris-HCl buffer solution pH 8.0. The total RNA without nongenetic code-based RNA enzyme was set-up as control. The seven tubes were thermo-cycled. Equal volumes of reaction products were briefly electrophoresed through ethidium bromide-stained 1% agarose gel in 1xMOPS buffer solution with formaldehyde to demonstrate the reaction complexes formed between GDH-synthesized nongenetic code-based RNA enzymes and total RNA (genetic code-based RNA).

The RNA enzymes synthesized by the acidic, mildly acidic, and neutral isoenzymes of laryngeal GDH degraded all the stomach total RNA (Figure 4). Since total RNA is a very complex mix of the transcriptome, RNA enzymes synthesized by GDH redox cycle isoenzymes must be equally complex in primary and secondary structures, diversity, copy numbers, and spatial/temporal population distribution patterns in order for them to degrade the entire transcriptome. The unprecedented diversity in the structure and population distribution of the GDH-synthesized RNA enzyme was demonstrated by the lack of defined banding patterns in the agarose gel in sharp contrast from total RNA (Figure 4).

4. Discussion

All genetic code-based RNAs (tRNA, rRNA, siRNA, microRNAs, mRNAs including those encoding transcription factors etc) are substrates for nongenetic code-based RNA enzyme synthesized by GDH redox cycle isoenzymes [16]. GDH-synthesized RNA enzyme is relevant in the alignment of environmental conditions with the biochemical pathways so that cells, tissues and the whole organism adjust their energy requirements according to the available environmental resources during programmed developmental, differentiation, growth, reproduction, and senescence transitions. Therefore, nongenetic code-base RNA enzyme is the fourth order in the biochemical hierarchy of the regulation of the genetic code; the first, second, and third orders being at the chromosomal DNA (replication, transcription), RNA (translation, and silencing by double-stranded RNA), and protein (enzymes) levels respectively (Figure 5).

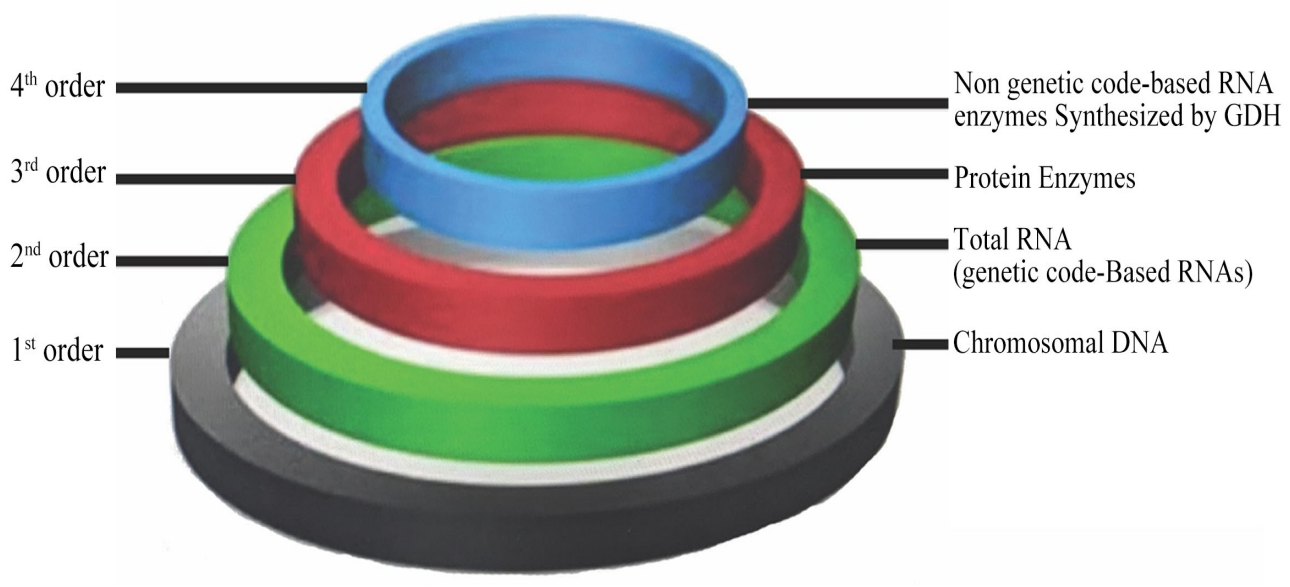

Figure 5. The expansiveness in molecular chemistry of substance dependency: Graphical representation of the foothold of GDH-synthesized nongenetic code-based RNA enzymes (4^{th} order); among other biochemical orders in the hierarchical sequence of dependency prognosis: protein enzymes (3^{rd} order), total RNA (2^{nd} order), and chromosomal DNA (1^{st} order).

The attack on the protonated N of the Schiff base complex formed between *α*-KG and the active site of GDH by strong nucleophiles is the committed step towards the degradation of GDH polypeptides, and for the *de novo* synthesis of GDH polypeptides. The new polypeptides may not be exactly the same as those degraded thereby creating a new pool of GDH redox cycle isoenzymes with properties to synthesize a new pool of nongenetic code-based RNA enzymes, which will recognize some of the pre-existing mRNAs, rRNAs, transfer RNAs, siRNAs etc as unnecessary and superfluous, and so degrade them. New biochemical pathways in alignment with the new chemical environment will arise in the cells, tissues, and organs creating a new biochemical variant of the organism. These are part of the biochemical changes that occur in people who smoke tobacco (nicotine as nucleophile), opium poppy (isoquiline alkaloids as nucleophile), cannabis (tetrahydrocannabinol as nucleophile), cocaine (neurocaine as nucleophile), or alcoholics (ethanol as nucleophile). These strong nucleophiles inactivate GDH polypeptides triggering changes in the population of the redox cycle isoenzymes, the nongenetic code-based RNA enzymes, mRNAs, proteins, and the biochemical pathways as described for the formation of new biochemical/metabolic variants in higher plant [13] [16].

Substance/chemical-dependent patients are therefore different biochemical/metabolic variants. This is why it is difficult for them to withdraw from addiction. People with chemical dependency continue to use narcotic drugs or alcohol, even knowing that continued use causes damages to their bodies, families, finances, and all other aspects of life. Chemical dependency is a primary disease in which a person becomes addicted to the narcotic drugs. Their bodies are chemically dependent on the narcotics because their biochemical pathways have

changed. Chemical dependency is a global medical and social pandemic, but its study has focused on the brain and central nervous system [23], and computational genomics [38]. There are more than 7000 different chemicals in tobacco, most of which are nucleophiles and hazardous. Consequently, more than 440,000 deaths occur yearly, caused by smoking and inhalation of narcotic smoke [19]. Smoking of opium poppy, cannabis, cocaine, etc causes similar pandemic-like chemical/substance dependency and fatality rates.

The degradation of cohorts of mRNAs that are homologous to the GDH-synthesized RNA enzyme may be indicative of the involvement of that mechanism [17] [18] also, in the biochemical progression of chemical dependency and diabetes. In peanut, the cohorts of mRNAs that described the ability of the crop species to produce the highest yields of seeds, amino acids, and fatty acids were up to 800, harshness of the environmental conditions notwithstanding [39] [40] [41]. In black eye beans, the cohorts of mRNAs that described the ability of the crop species to produce the highest dry grain yield were more than 60, harshness of the environmental conditions notwithstanding [16]. The ability of human GDH-synthesized RNA enzyme to degrade human total RNA (**Figure 4**) as in plants indicates that the RNA enzyme will degrade cohorts of homologous mRNAs encoding different enzymes in different biochemical pathways. Cohorts of mRNAs that describe the abilities of plant species to survive environmental conditions are analogous to cohorts of mRNAs that describe human ability to die from chemical dependency disease conditions. These are some of the differentiations that GDH-synthesized nongenetic RNA enzyme introduces into molecular chemistry for the study of the genome in cohorts of mRNA-causing diseases in addition to the nucleotide sequences of the mRNAs. Whereas mRNAs, transfer RNAs, rRNAs etc (**Figure 5**) are electromagnetic reflections of the genome; proteins are also electromagnetic mirror images of mRNAs; the GDH-synthesized RNA enzymes being different because they are not related to the chromosomal DNA coding template. It is not always the case that a disease condition is caused by the malfunctioning of a single gene. What makes a person an alcoholic, or what leads to chemical dependency may not be the malfunctioning of a single gene, but likely to include the malfunctioning of a cohort of mRNAs under the control of GDH redox cycle isoenzymes. Degradation of mRNAs by their homologous GDH-synthesized RNA enzyme is a statistical chemical reaction that is dependent on the structure, relative concentration, and temporal distribution of both substrate and enzyme molecules [40] [41]. The etiology and prognosis for alcoholism and drug abuse are complex because in addition to inhibition of neurotransmission in the nervous system by narcotics [1]-[6] [19] [20], and the potential alteration of the biochemical pathways that are related to GDH synthesis of nongenetic code-based RNA enzyme, emerging computational genomics research results suggest that the noncoding section of chromosomal DNA is involved in the enhancement of eating disorders [38]. This adds more complexity to an already inexplicable substance abuse pheno-

menon. Nongenetic code-based RNA enzyme (4^{th} order), protein enzymes (3^{rd} order), total RNA (2^{nd} order), and chromosomal DNA (1^{st} order) are therefore integral participants that are at high risk of being aggravated by narcotic drugs in the hierarchical progression, regulation, and management of substance dependency (**Figure 5**).

Therefore, the innovative advantage of the application of molecular chemistry in the study of biological phenomena is that the cohorts of mRNAs that are degraded by their homologous nongenetic code-based RNA enzymes are different from the transcriptional and protein networks that are studied via computational and mathematical modeling [42] [43] because mRNA cohorts are experimental data for studying the integration/discrimination regulation of biochemical pathways; they are based on chemistry, an exact science devoid of false positive and false negative results. The degradation of superfluous mRNAs (**Figure 4**) by nongenetic code-based RNA enzyme synthesized by NADH-GDH followed by the coordination of several biochemical pathways was first described as the signal discrimination/integration function based on the amination Michaelis constants of GDH [14]. The house keeping cyclical redox transformation of α-KG to L-glu is the homeostasis environment [16] for the activation of the hexameric isoenzymes of NADH-GDH to synthesize plus-RNA in the amination, but minus-RNA in the deamination direction (**Figure 1**). The synthesis of new pools of nongenetic code RNA enzyme, and degradation of their homologous superfluous mRNAs consequent upon changes in the nucleophilic chemical environment of NADH-GDH create the differentiating parameters for the accurate modeling of the responses of biochemical pathways to chemical/substance over dose and addiction disease conditions.

The foregoing renders it imperative that intervention and treatment approaches for substance dependency need to be complex, elaborate, and inclusive of medical, molecular chemistry, biotechnological, computational, sociological, and educational inputs in order for positive outcomes to be achieved.

5. Conclusion

Human GDH redox cycle isoenzyme population distribution pattern obtained through electrophoretic purification is in agreement with the GDH_1 and GDH_2 gene structure of the enzyme, the GDH-synthesized RNA enzymes spanning wide range (25 bases - 20K bases) molecular weight. Prep Cell polyacrylamide gel purification confirmed that Rotofor isoelectric focusing produced pure GDH redox cycle isoenzymes, which procedure may become an innovative approach and paradigm shift for biochemical diagnosis of nicotine, cannabinoid, opioid, cocaine overdose and addiction. Accurate diagnosis of a disease condition leads to appropriate procedures for management of the disease condition.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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